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Signal Transduction of the TGF-β Superfamily by Smad Proteins

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Members of the TGF-β superfamily regulate the growth and differentiation of various types of cells. Smads are recently identified proteins that mediate intracellular signaling of the TGF-β superfamily. Smads are grouped into three classes depending on their structure and functions. R-Smads are phosphorylated by type I serine-threonine kinase receptors for TGF-β superfamily members. R-Smads then associate with Co-Smads. Smad4 is the only vertebrate Co-Smad identified thus far, and is required for the signaling pathways of different ligands. The heteromeric Smad complex translocates into the nucleus, where it activates target genes. Anti-Smads inhibit signaling by R-Smads and Co-Smads. Smads bind to DNA directly or indirectly *via* other DNA binding proteins. R-Smads interact with transcriptional coactivators, and have intrinsic transactivation activity. Elucidation of the functions of Smads will provide the framework for research on TGF-β superfamily signaling.

Key words: DPC4, Mad, serine-threonine kinase receptor, Smad, TGF-β.

Members of the transforming growth factor-β (TGF-β) superfamily are growth/differentiation factors that regulate a wide range of fundamental biological processes in multicellular organisms. Over forty members of this family have been isolated, and TGF-βs, activins/inhibins, and bone morphogenetic proteins (BMPs) form three major subfamilies (reviewed in Refs. *1* and *2*). TGF-β1 was originally identified as a secreted factor that transforms normal cells in combination with other growth promoting factors. TGF-β1, however, was subsequently shown to be a potent growth inhibitor of various cell lineages including epithelial, hematopoietic, and endothelial cells. TGF-β1 also induces the expression of extracellular matrices and cell adhesion molecules, thereby controlling cell communication. Activins/inhibins were identified as regulators of the production of pituitary follicle-stimulating hormone (FSH). Activins also promote the differentiation of erythroid cells. In *Xenopus laevis*, activins are essential mesoderm inducers. BMPs were purified as factors that induce ectopic bone formation. Like activins, BMPs regulate various early developmental processes in invertebrates and vertebrates.

Such diverse functions of the TGF-β superfamily have attracted the attention of researchers in various fields of biological science, and extensive efforts have been made to elucidate the signaling pathway of the superfamily. Signals of TGF-β-related factors are propagated across the cell membrane by two types of the serine-threonine kinase receptors termed type I and type II. Conversely, all known ligands for serine-threonine kinase receptors belong to the TGF-β superfamily. The type II receptor is a constitutively active kinase, whereas the type I receptor is inactive in the absence of ligand. Similar to the activation of tyrosine kinase receptors through dimerization, ligand binding induces heteromeric oligomerization of the type I and type II receptors, resulting in transphosphorylation of the type I receptor by the type II kinase. The type I receptor then phosphorylates recently discovered intracellular target molecules.

The signal transduction pathway of the TGF-β superfamily is highly conserved through invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* to vertebrates including amphibians and mammalians. Decapentaplegic (Dpp) is a BMP homolog in *Drosophila*. A genetic screen for enhancers of weak *dpp* alleles resulted in the isolation of *Mothers against dpp* (*Mad*) as a gene involved in the signaling pathway of Dpp (*3*). Mad was a novel molecule with no homology to known proteins. Subsequent genetic studies in *Drosophila* demonstrated that Mad acts downstream of a Dpp receptor. Daf-4 is a type II receptor for a BMP-like ligand in *C. elegans*. *sma-2, 3*, and *4* were identified as genes that exhibit phenotypes similar to those of *daf-4* mutations (*4*). Strikingly, the protein structures of the three *sma* genes were significantly similar to that of *Mad*. These independent results in two different organisms strongly indicated that molecules related to Mad and Sma mediate intracellular signaling of the TGF-β superfamily.

The first mammalian member of the new protein family was identified in a search for a tumor suppressor gene (*5*). Chromosome 18q21 is frequently mutated in pancreatic cancers, and genomic analyses of the affected region revealed *homozygously deleted in pancreatic carcinoma, locus 4* (*DPC4*) as a candidate tumor suppressor. DPC4 was shown to be homologous to Mad and Sma in amino acid sequence. An increasing number of vertebrate homologs of Mad and Sma have since been identified primarily by homology

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search of the expressed sequence-tag (EST) database, and are now generically denoted as Smad, the term coined from Sma and Mad.

Eight mammalian Smads have been reported (reviewed in Ref. *6*) and grouped into three classes based on their structure and functions (Fig. 1). Pathway-restricted or receptor-regulated Smads (R-Smads) are directly phosphorylated by type I receptors. Smad2 and Smad3 are substrates of TGF-β and activin receptors, whereas Smad1, Smad5, and possibly Smad8, propagate BMP-specific signals. In contrast, DPC4 or Smad4, which belongs to the second class, is a common mediator (Co-Smad) required by all distinct pathways. Smad4 is the only vertebrate Co-Smad identified thus far. Phosphorylated R-Smads form heteromeric complexes with Co-Smads, translocate into the nucleus, and activate a specific set of genes (Fig. 2). Smads in the third class (Anti-Smads) antagonize signaling by R-Smads and Co-Smads. Smad6 (*7, 8*) and Smad7 (*9, 10*), which belong to this class, inhibit TGF-β/activin and/or BMP signaling. Three classes of Smads have also been identified in *Drosophila* (Fig. 1) (reviewed in Ref. *11*). Mad, the founding member of the Smad family, is an R-Smad that is closely related to Smads 1, 5, and 8, consistent with its specificity to the signaling pathway for Dpp, a BMP-like ligand. Medea is a Co-Smad, whereas Dad is an Anti-Smad.

R-Smads share two conserved regions, the MH (Mad homology) 1 domain in the N-terminal part and the MH2 domain in the C-terminal part, separated by a middle linker region varying in sequence and length (Fig. 1). R-Smads are distinguished from the other Smads by an SSXS (Ser-Ser-X-Ser) motif at the C-terminal end. The last two serines of the motif are the direct phosphorylation sites by type I receptors (*12, 13*). Co-Smads contain the MH1 and MH2 domains, but not the SSXS motif, and are not phosphorylated by type I receptors. Anti-Smads, however, share only the MH2 domain while their N-terminal half diverges from the conserved MH1 domain.

Molecular functions of Smads

Activation of Smads. The direct evidence that Smads act downstream of serine-threonine kinase receptors came from biochemical studies. Smads 1, 2, and 3, later categorized as R-Smads, interact directly with type I receptors upon ligand binding, and undergo phosphorylation by the receptor kinases (*14-16*). The binding of R-Smads to type I receptors requires transphosphorylation of the type I receptors by type II receptors. When the kinase activity of the type II receptor is rendered inactive by mutation,

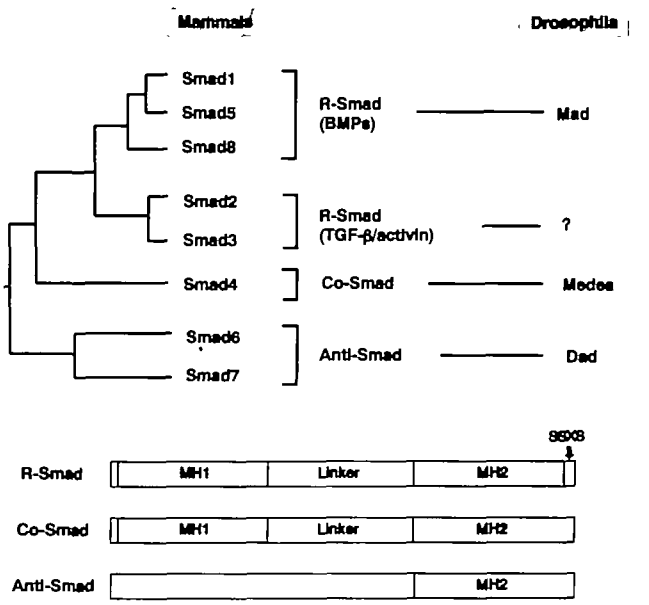

Fig. 1. **Classification and structures of Smads.** Smads are grouped into three classes depending on their structure and functions. Mammalian and *Drosophila* Smads are listed. R-Smads are receptor-regulated Smads that contain the MH1 and MH2 domains and the SSXS motif. Co-Smads are common mediators with MH1 and MH2, but lack the SSXS motif. Anti-Smads inhibit signaling, and the structures are greatly diverged from those of the other Smads.

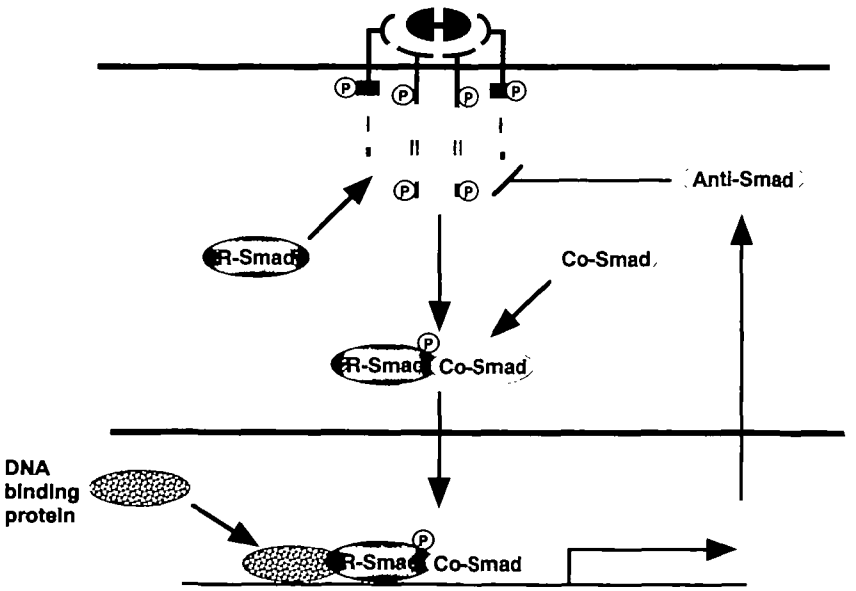

Fig. 2. **Smad signaling.** R-Smads are phosphorylated by type I receptors upon ligand binding. R-Smads then associate with Co-Smads and translocate into the nucleus. The Smad complexes bind to DNA either indirectly through interaction with other DNA binding proteins or directly, and transactivate target genes.

R-Smads do not associate with the type I receptors even in the presence of ligand (*16*). The interaction of R-Smads with type I receptors is transient, which is consistent with the physiological function of Smad proteins in conveying signals from the cell membrane to the nucleus. Either modification of the SSXS motif in R-Smads or disruption of the kinase activity of type I receptors allows the detection of a stable Smad-receptor interaction (*16*), because these manipulations abrogate phosphorylation but not interaction. The critical region in Smad1 and Smad2 required for their specific interaction with TGF-β type I receptor (TβR-I/ALK5) or BMP type IB receptor (BMPR-IB/ALK6) was determined under such conditions (*17*). The interaction was mapped to the "L3 loop" that was identified in the crystal structure of the MH2 domain of Smad4 (*18*). The amino acid sequence of the L3 loop is highly conserved except for two amino acids between TGF-β-regulated Smads (Smads 2 and 3) and BMP-responsive Smads (Smads 1, 5, and 8) (Fig. 3). Exchange of these two amino acids between Smad1 and Smad2 switches the signaling specificity of the Smads including receptor interaction and phosphorylation (*17*).

Upon binding to type I receptors, R-Smads undergo phosphorylation. The phosphorylation site is the C-terminal SSXS motif characteristic of R-Smads. Replacement of the serines in the SSXS motif with alanines causes disruption in the functions of Smad1 (*19*), Smad2 (*13, 16*), and Smad3 (*20*). Furthermore, the mutated Smad2 and Smad3 interfere with the signaling of TGF-β, acting in a dominant-negative fashion. Interestingly, alteration of the serines to aspartic acids, which mimic the negative charge of phosphate, does not cause full activation of Smad3, but a significant increase in the basal activity in the absence of ligand stimulation (*20, 21*). Phosphorylation of the SSXS motif seems to induce conformational changes in R-Smads, which could result in their activation. The MH1 and MH2 domains interact with each other (*22*), and removal of the MH1 domain induces transactivation by the MH2 domain (*23*), whereas MH2 inhibits the DNA binding ability of MH1 (*24*). Thus the MH1 and MH2 domains repress each other through intramolecular interaction, and phosphorylation relieves this mutual repression (Fig. 4).

Phosphorylation of R-Smads induces the formation of complexes of R-Smads and Co-Smads. Several lines of evidence suggest that Smad4 is an essential common mediator in both TGF-β/activin and BMP signaling pathways. Smad4 and Smad3 activated a TGF-β-responsive luciferase reporter in a synergistic manner (*15*). Smad4 has been shown to be required for both Smad1- and Smad2-mediated mesoderm induction in *Xenopus* embryos (*25*). A breast cancer cell line lacking Smad4 is unresponsive to TGF-β, and the introduction of exogenous Smad4 into the cells restored growth inhibition by TGF-β (*26*). The molecular mechanism underlying the Smad4 requirement is illustrated by the physical association of Smad4 with R-Smads upon ligand stimulation (*25*) (Fig. 2). Smad4 interacts with Smad1 in the presence of BMP and with Smad2 in response to TGF-β or activin. Thus the heteromeric complexes of R-Smads and Co-Smads are likely to be the active forms of Smads. Interestingly, however, Smad2 and Smad3 also form a complex in a ligand-dependent manner (*27*), and the ligand-induced homo-oligomerization of R-Smads was recently reported (*28*). All three classes of Smads including R-Smads, Co-Smad, and Anti-Smads exist as monomers in the absence of ligand. TGF-β stimulation induces homo-oligomerization of Smad2 or Smad3 as well as hetero-oligomerization of the R-Smads with Smad4. Crystal structure analysis of the Smad4 MH2 domain revealed Smads to have the ability to form trimers (*18*). Thus the homo- and hetero-oligomers are likely to be trimers, although a direct demonstration awaits further investigation. Homo-oligomeric Smad3 binds to DNA as the heteromeric Smad3-Smad4 complex does (*28*), and may play an as yet unknown role in transactivation.

The heteromeric complexes of R-Smads and Co-Smads translocate into the nucleus. R-Smads have been shown to move into the nucleus in the absence of Co-Smads, whereas Co-Smads require R-Smads to accumulate in the nucleus (*29, 30*). Thus R-Smads are the driving force for nuclear translocation. However, no typical nuclear location signal (NLS) is found in the amino acid sequence of R-Smads. The MH1 domain of Smad3 contains a stretch of basic amino acids, which may function as an NLS, expressed on the surface of the protein, as determined by crystallization of the MH1 domain of Smad3 (*31*). However, deletion of the MH1 domain and the linker region causes nuclear accumulation of Smad2 in *Xenopus* embryos (*32*). The MH2

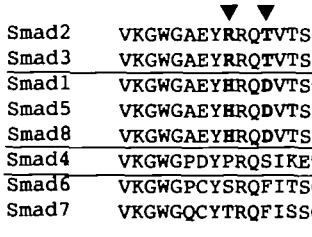

Fig. 3. **Sequence alignment of the L3 loop of mammalian Smads.** Sequences of the L3 loops of mammalian Smads are compared. This region confers signaling specificity to R-Smads. Only two amino acids (in bold type and indicated by arrowheads) differ between TGF-β/activin-responsive Smads (2 and 3) and BMP-specific Smads (1, 5, and 8).

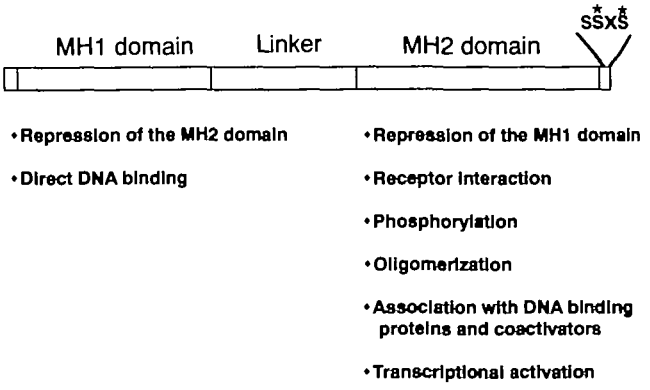

Fig. 4. **Functions of the MH1 and MH2 domains of R-Smads.** The functions of the MH1 and MH2 domains are summarized. The two regions repress each other through intramolecular interactions. The last two serines (with asterisks) of the SSXS motif are phosphorylated by type I receptors.

domain may thus contain a signal for nuclear translocation and/or the MH1 domain may harbor a cytoplasmic retention signal. The precise mechanism of nuclear translocation of Smad proteins remains to be determined. In *Drosophila*, overexpression of Dpp causes the nuclear accumulation of Mad (*33*). Under physiological conditions, however, Mad localizes in the cytoplasm, even in the region where Dpp activity is high (*33*). An undetectable level of nuclear accumulation of Mad may be sufficient for the propagation of Dpp signals *in vivo*.

Antagonistic and dominant-negative Smads. Anti-Smads joined the Smad family most recently. The mechanisms whereby Anti-Smads exert their inhibitory effects have been investigated in the mammalian system. Smad6 and Smad7 associate stably with type I receptors, thereby inhibiting the phosphorylation of R-Smads (*7-10*). In BMP signaling, Smad6 may also compete with Smad4 in association with Smad1 (*8*). *Daughters against dpp* (*Dad*) was identified as a gene whose expression is induced by Dpp. Dad is structurally similar to the vertebrate Anti-Smads, and antagonizes Dpp signaling (*34, 35*). The expressions of Dad, Smad6, and Smad7 are induced by ligands, and the auto-regulatory feedback loop *via* Anti-Smads seems to be conserved between invertebrates and vertebrates (Fig. 2) (*10, 34, 36, 37*).

Missense mutations of Smad4 and Smad2 have been reported in various malignant tumors. Many of the mutations are located at the interface between the monomers in the trimeric model of Smad4, suggesting that oligomerization is essential to Smad function (*18*). Alteration of the conserved aspartic acid-450 to glutamic acid results in the loss of function of Smad2 (*38*). The corresponding mutation was introduced into Smad3 and characterized (*39*). Mutant Smad3 is not phosphorylated by TβR-I, and, furthermore, associates stably with TβR-I and interferes with the phosphorylation of wild type Smad2 and Smad3 as Anti-Smads do. However, in contrast to Anti-Smads, the inhibition is specific to TβR-I, and mutant Smad3 does not block the phosphorylation of Smad1 by BMPR-IB. The mutation thus not only disrupts the normal function of wild type Smad, but creates a dominant-negative Smad that could actively contribute to oncogenesis.

Nuclear functions of Smad. Members of the TGF-β superfamily regulate the transcription of various genes including cell cycle regulators, extracellular matrices, adhesion molecules, homeobox genes, and ligands themselves. This broad range of target genes reflects the multifunctional nature of the ligands. Smads are the messengers that directly convert phosphorylation at the cell membrane to gene expression in the nucleus. The role of Smads as transcriptional regulators was initially revealed by utilizing the fusion proteins of Smads with the yeast DNA binding protein, GAL4 (*23*). The GAL4 fusions of the full length Smad1 and Smad2 activate the expression of a reporter with GAL4 binding sites in response to BMP and TGF-β, respectively. Moreover, the fusion of the MH2 domain constitutively induces transactivation. Thus, the MH2 domain has an intrinsic transactivation activity, and the MH1 domain has a regulatory function. Phosphorylation relieves this intracellular regulation as discussed above.

Mix.2 is an early response gene induced by activin in *Xenopus* embryos. The 50 bp activin responsive element (ARE) in the *Mix.2* promoter was determined, and one-hybrid screening with ARE resulted in the identification of a novel transcription factor with a winged-helix/forkhead motif, FAST-1 (*40*). FAST-1 interacts directly with Smad2 in an activin-dependent manner (*29, 41*). Smad4 is incorporated in the transcription complex, and promotes DNA binding through its MH1 domain and activates transcription through its MH2 domain (*29*). The FAST-1 interaction domain within the MH2 domain of Smad2 has been determined (*42*), and exchange of this region between Smad1 and Smad2 causes the interaction of Smad1 with FAST-1 in response to BMP.

Smads are unique in DNA binding. They are tethered to DNA not only *via* interaction with other DNA binding proteins such as FAST-1, but also bind directly to specific DNA sequences. *vestigial*, *labial*, and *ultrabithorax* are Dpp-responsive genes in *Drosophila*. Mad was shown to bind directly to the promoter regions of these genes (*24*) with a consensus binding sequence of GCCGNCGC. The MH1 domain binds to DNA and the MH2 domain inhibits the binding. Another recent study using *Drosophila* demonstrated that Medea binds directly to DNA as well (*43*). *tinman* is a Dpp-inducible homeobox gene that plays a pivotal role in mesoderm induction. Two Dpp-responsive elements were identified in the 349 bp regulatory region of the gene (*43*). One-hybrid screening with one of the Dpp-responsive elements pulled out Medea as a binding protein. Footprinting analysis using GST-Smad fusion proteins disclosed multiple Mad and Medea binding sites in the 349 bp enhancer region. The consensus sequence of these binding sites is CGCCGC(G/C)G(C/A)C, which is almost identical to the previously reported "GCCG" motif in *vestigial*. One intriguing finding is that the Mad/Medea binding site is not sufficient for the expression of *tinman*. The adjacent "GAATGT" sequence, which is closely related to the FAST-1 binding sequence (*44*), is required for the expression, suggesting the existence of another essential transcriptional cofactor.

Smad4 has been shown to bind directly to a specific sequence around an AP-1 site in the collagenase promoter (*45*). Screening of Smad3 and Smad4 binding sites using gel shift-PCR selection of oligonucleotides identified an palindromic sequence GTCTAGAC as a consensus binding sequence (SBE, for Smad binding element) (*46*). *Plasminogen activator inhibitor-1* (*PAI-1*) and *JunB* are TGF-β-responsive genes. Sequence examination of both promoters resulted in the identification of the CAGACA motif as a common binding site for Smad3 and Smad4 (*47, 48*). SBE and the CAGACA motif have a significant overlap of GTCT or its complimentary AGAC sequence. Concatamerization of these sequences confers TGF-β responsiveness to a heterologous promoter reporter, suggesting that DNA binding of Smads may be sufficient for transactivation by TGF-β. Notably, however, reporter genes with a low number of copies of the Smad binding sites respond only minimally to TGF-β or Smad, as will be discussed later.

Recent determination of the crystal structure of the MH1 domain of Smad3 bound to SBE revealed the three-dimensional configuration of the DNA binding of Smad3 (*31*). A conserved β hairpin structure is embedded in the major groove of the target DNA. Smad2 and Smad3 are highly similar in structure, however, the direct DNA binding of Smad2 has not been detected. One of the differences

between Smad2 and Smad3 is the 30 amino acid insertion in the MH1 domain of Smad2, and the removal of this region endows Smad2 with DNA binding capability (*49*). This region may sterically interfere with the β hairpin in DNA binding.

Human FAST-1 (hFAST-1), which is closely related to *Xenopus* FAST-1 in its forkhead domain, has been identified (*44*). The PCR-based strategy used in the determination of SBE (*46*) was employed to define the DNA motif for hFAST-1 binding, whose consensus sequence was revealed to be TGT(G/T)(T/G)ATT (FBE, for FAST-1 binding element). The sequence is contained in the *Xenopus Mix.2* gene to which *Xenopus* FAST-1 binds (*40*). Reporter genes with either a single FBE or SBE do not respond to TGF-β, however, a reporter with a combination of FBE and SBE responds to TGF-β in the presence of hFAST-1. Interestingly, the *Mix.2* gene also contains two CAGA motifs around the FAST-1 binding site. The results thus indicate that both sites may be required for the activation of FAST-1-responsive genes.

FAST-2, another mammalian homolog of FAST-1, induces the expression of *goosecoid*, an activin-responsive gene expressed in gastrulation and mesoderm induction (*50*). FAST-2, like FAST-1, interacts with Smad2 in a ligand-dependent manner. FAST-2 activates a reporter gene with an activin responsive element from *goosecoid* in the presence of Smad2 and Smad4. The binding sites for FAST-2 and Smad4 in the *goosecoid* gene were determined by footprinting analysis. FAST-2 binds to AATCCACA, which is identical to FBE (*44*). Smad4 binds to GC-rich sequences around FBE where four GCCG motifs exist. FAST-2 binds to DNA constitutively, whereas Smads require FAST-2 for DNA binding, suggesting that the affinity of Smads for DNA is relatively low. Smad3 in this system blocks the transactivation of the gene, but the precise mechanism is still unclear. Thus, FAST-2 recruits the Smad complex to a specific site on the DNA and Smads activate transcription. Similar cooperative binding between Smads and other DNA binding proteins has been reported in the case of *tinman* (*43*) and AP-1 (*51*).

A growing number of transcription factors interact with coactivators such as p300 and CBP to induce gene expression. Smads were recently shown by several groups to interact directly with p300/CBP (*21, 52-55*). Smad2 and Smad3 interact with p300/CBP through the MH2 domain, which is consistent with its inherent transactivation activity. p300/CBP associates with various DNA binding proteins including c-Jun and c-Fos, and may thus act as a bridging factor between Smads and other transcription factors. E1A, an adenoviral oncoprotein, blocks transactivation by TGF-β, and the inhibition is likely to be mediated through its interaction with coactivators.

The molecular functions of the MH1 and MH2 domains are summarized in Fig. 4, and the current model of transactivation by Smads is illustrated in Fig. 5. Smads have an intrinsic DNA-binding ability, but the affinity is relatively low. Thus the concatamerization of the binding sites enhances the Smad-responsiveness of an artificial reporter. Under physiological conditions, sequence-specific DNA binding proteins recruit Smad complexes to the DNA. This allows the interaction of Smads with adjacent binding sites on the DNA. The multiple protein-DNA interactions then support the stabilization of the higher-order DNA-binding

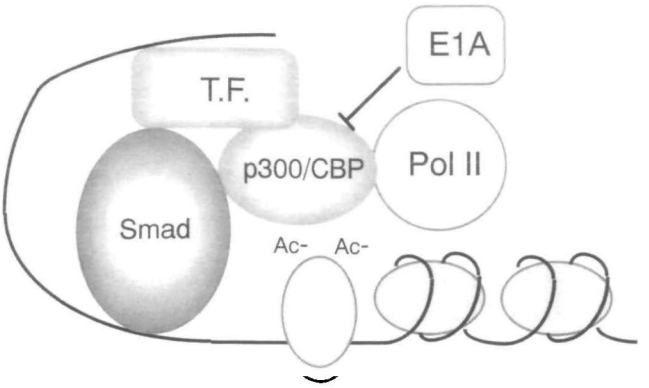

Fig. 5. **Transactivation by Smads.** Smads are tethered to DNA through interactions with other sequence-specific DNA binding transcription factors (T.F.). Smads stabilize the DNA binding by binding directly to adjacent sites on the DNA. Smads recruit transcriptional coactivators such as p300/CBP. These coactivators interact directly with RNA polymerase II and "melt" DNA with their histone acetyltransferase activity, thereby activating transcription. E1A, an adenoviral oncoprotein, inhibits transcription by binding to coactivators.

complexes. Smads induce the expression of the target gene at least through interaction with coactivators such as p300/CBP. Coactivators may further stabilize the transactivation complex through their multiple interactions.

Signaling cross talks through Smads

Members of the TGF-β superfamily act in environments where multiple signals interact, and thus are likely to cross-talk with other signaling pathways. Calmodulin, a central molecule in calcium signaling, interacts with Smads in a calcium-dependent manner *in vitro* (*56*). Although inhibitors of calmodulin enhance the activation of the TGF-β/activin-responsive p3TP-Lux reporter by activin, overexpression of calmodulin inhibits the activation of p3TP-Lux. Further investigations are needed to determine the physiological relevance of the Smad-calmodulin interaction. EGF induces the phosphorylation of the linker region of Smad1 through activation of the ERK kinase, and inhibits its translocation from the cytoplasm to the nucleus. EGF may thus antagonize BMP signaling through Smad1 (*57*). In another report, however, EGF and HGF were shown to positively regulate Smad signaling (*58*). HGF induces the phosphorylation of Smad2, probably through MEK1, and stimulates the activation of p3TP-Lux. The phosphorylation site has not been determined, and the molecular basis of the positive regulation remains to be shown. Both synergistic and antagonistic regulations between the TGF-β superfamily and the MAPK cascade are known (*11*), and Smads may be the site of the intersection.

Roles of Smads *in vivo*

The roles of Smads *in vivo* have been investigated by gene targeting in mice. Smad4 knockout mice died at an early embryonic stage (before day 7.5) and exhibited defects in visceral endoderm differentiation, gastrulation, and mesoderm induction, indicating that Smad4 plays a pivotal role in early development (*59, 60*). Further analyses, however, revealed an unexpected aspect to the Smad4 functions. The gastrulation defect was rescued by forming chimeras of the mutant ES cells with wild type cells,

suggesting that the primary requirement of Smad4 in gastrulation does not reside in the embryo but in the extraembryonic tissues. This result is in prominent contrast to BMP-4 or BMP type IA receptor (BMPR-IA/ALK3) knock-out mice that died from defects in gastrulation and mesoderm formation. Thus the results of the Smad4 targeting suggest the possibility of a novel role for the TGF-β superfamily in early development.

Smad2 targeting has been reported by three groups (*61-63*). Smad2 knockout mice died at early stages of development, showing a lack of embryonic germ layers and defects in the determination of the anterior-posterior polarity. However, the phenotypes differed significantly in other aspects. In one report, the extraembryonic tissues developed relatively normally, and the entire epiblast, the prospective embryo, differentiated to extraembryonic mesoderm (*61*). In the other reports, however, the mutant mice lacked the extraembryonic portion as well as the mesoderm. These opposing results may be explained by differences in the targeting strategies. The results also demonstrate that Smad2 and Smad3 are not redundant in early development. In remarkable contrast to Smad2, Smad3 null mice were viable and fertile (*64*). The mice developed metastatic colorectal cancers, indicating that Smad3 is a tumor suppressor. The results again confirm the non-redundant roles of Smad2 and Smad3 *in vivo*.

As Smad4 knockout mice died as embryos, it was not possible to investigate the role of Smad4 in carcinogenesis using homozygous mutants. Mice with concomitant heterozygous mutations of *Smad4* and *Apc*, a gene responsible for familial adenomatous polyposis, were established (*65*). The compound mice developed more malignant tumors than simple *Apc* heterozygotes, suggesting that mutations in Smad4 contribute to the malignant progression of cancers. Recently, germ line mutations of *Smad4* were reported in a subset of juvenile polyposis (*66*). Studies of mouse models and diseases will extend our understanding of the role of Smads in tumor suppression.

Conclusions

Smads comprise a novel family of signaling mediators downstream of serine-threonine kinase receptors. They transmit signals directly from the cell surface to the nucleus. R-Smads and Co-Smads cooperate in positive signaling, whereas Anti-Smads are inhibitory. The biochemical characterization of these Smads has brought about remarkable achievements in the elucidation of the intracellular signaling pathway of TGF-β-like ligands. However, important questions remain. For example, what is the mechanism for the nuclear accumulation of Smads? Smad3 can bind directly to DNA in the absence of Smad4, and Smad3 interacts with coactivators. In that case, why do R-Smads require Co-Smad in transactivation? How is signaling specificity determined by various combinations of receptors, Smads, and DNA binding partners? Answers to these and other remaining questions will lead to a full understanding of the molecular mechanisms of TGF-β superfamily signaling.

The recent advances in the study of Smads, however, do not exclude the direct involvement of other signaling pathways. In fact, TGF-β has been shown to activate components of the MAP kinase cascade (*67*). Type I or even type II receptors may phosphorylate other yet unidentified signaling components. Also, Smads, including Anti-Smads, may have additional functions. We need to recognize that non-Smad or non-transcriptional regulation by TGF-β-related factors has not been fully studied, and leave doors open to these possibilities. Nevertheless, the identification of the Smad family provides the framework for the future research in this field.

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