Specificity and Versatility in TGF-β Signaling Through Smads

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Key Words

TGF-β receptor, bone morphogenetic protein, signal transduction, complexity, transcription

Abstract

The TGF-β family comprises many structurally related differentiation factors that act through a heteromeric receptor complex at the cell surface and an intracellular signal transducing Smad complex. The receptor complex consists of two type II and two type I transmembrane serine/threonine kinases. Upon phosphorylation by the receptors, Smad complexes translocate into the nucleus, where they cooperate with sequence-specific transcription factors to regulate gene expression. The vertebrate genome encodes many ligands, fewer type II and type I receptors, and only a few Smads. In contrast to the perceived simplicity of the signal transduction mechanism with few Smads, the cellular responses to TGF-β ligands are complex and context dependent. This raises the question of how the specificity of the ligand-induced signaling is achieved. We review the molecular basis for the specificity and versatility of signaling by the many ligands through this conceptually simple signal transduction mechanism.

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INTRODUCTION

Members of the TGF-β family are secreted polypeptides that activate cellular responses during growth and differentiation. More than 60 TGF-β family members have been identified in multicellular organisms, with at least 29 and probably up to 42 proteins encoded by the human genome. Among these 60, there are three TGF-βs, five activins, and at least eight BMPs encoded by different genes.

TGF-β: transforming growth factor-β

TGF-β-related factors are made as precursors with a large propeptide and a C-terminal mature polypeptide that is proteolytically cleaved from the precursor (Annes et al. 2003). Mature TGF-β is a homodimer of two 12.5-kd polypeptides joined by a disulfide bond. Two copies of the propeptide remain associated with the TGF-β and maintain it in an inactive complex known as LTBP. An LTBP is often linked to the prosegment and plays a role in targeting the complex to the extracellular matrix, where TGF-β is activated and released by proteolytic cleavage of the prosegment (Annes et al. 2003). Other TGF-β family members are also expressed as disulfide-linked homodimers or heterodimers, are likely secreted as complexes, and undergo proteolytic activation. The activities of TGF-β family members are often regulated by secreted and matrix-associated proteins that bind the ligands in solution, thus sequestering the ligands from access to their receptors or helping to ensure ready availability (Annes et al. 2003). In addition, several cell surface proteins function as coreceptors and help in the presentation of the ligand to the receptor.

TGF-β family members are expressed in most cell types and play key roles in differentiation and tissue morphogenesis. TGF-β itself inhibits proliferation of many cell types, including epithelial and hematopoietic cells, and its signaling controls tumorigenesis. The cell's responses to TGF-β are complex as a result of differential transcriptional regulation and nontranscriptional effects that depend on the cell context and physiological environment.

The cell surface receptors for TGF-β-related proteins and Smads as intracellular effectors of TGF-β responses have been identified, and the general scheme for signaling from the cell surface to the nucleus has been established (Derynck & Zhang 2003, Shi & Massagué 2003). In addition, receptor activation induces non-Smad signaling

pathways that can regulate Smad signaling or lead to Smad-independent responses (Derynck & Zhang 2003). This review focuses on the question of how the Smad signaling mechanism, comprised of a limited number of receptors and Smads and a multiplicity of TGF-β ligands, exerts specificity and at the same time displays a considerable versatility in cellular responses.

THE GENERAL MODEL OF TGF-β-INDUCED SIGNALING THROUGH SMADS

TGF-β proteins signal through cell surface complexes of "type I" and "type II" receptors. These two types are structurally similar transmembrane serine/threonine kinases, but type I receptors have a conserved Gly/Ser-rich "GS sequence" immediately upstream from the kinase domain. Ligand binding allows the formation of a stable receptor complex consisting of two receptors of each type, allowing phosphorylation of the GS sequences by the type II receptor kinases. This phosphorylation activates the type I receptor kinases, resulting in autophosphorylation of the type I receptor and phosphorylation of Smad proteins (**Figure 1**) (Derynck & Zhang 2003, Shi & Massagué 2003).

The Smads are the only established intracellular effectors of TGF-β signaling. Smads exist as three subgroups: R-Smads, a common Smad (e.g., Smad4 in vertebrates), and inhibitory Smads. R-Smads and Smad4 contain two conserved polypeptide segments, the MH1 (N) and MH2 (C) domains linked by a less conserved linker region. The R-Smads have a C-terminal SXS motif in which both serines are targeted for direct phosphorylation by the type I receptors. Thus, upon ligand binding, the type I receptors recruit and phosphorylate R-Smads, i.e., Smad2 and Smad3, by the TβRI/ALK-5 type I receptor in response to TGF-β, and Smad1, Smad5, and Smad8 by BMP type I receptors. C-terminal SXS phosphorylation of the R-Smads leads to their conformational changes, their dissociation from the type I receptors, and the formation of a trimeric complex consisting of two R-Smads and one Smad4. This trimeric complex translocates into the nucleus, where the Smads act as transcription factors (Derynck & Zhang 2003, Shi & Massagué 2003).

Smads act as ligand-induced transcription regulators of TGF-β responses. At the regulatory DNA sequences of genes, Smads activate transcription through assembly of a large nucleoprotein complex consisting of Smad-binding DNA elements, DNA-binding transcription factors, and the transcriptional coactivators. R-Smads and Smad4 have weak intrinsic DNA-binding ability and exhibit less stringent sequence requirements than the Smad-interacting transcription factors, which have high-affinity binding to a specific DNA sequence. Thus, a Smad-binding sequence in proximity to the cognate sequence for the interacting transcription factor allows for Smad-mediated transcriptional regulation. This mechanism explains why TGF-β activates only a select set of promoters with binding sites for the interacting transcription factor. The Smad interactions with coactivators CBP/p300 allow the Smad complex to enhance the inherent transcription activity of the interacting transcription factor(s). Therefore, the Smad complex may be considered as a coactivator complex for select transcription factors (Derynck & Zhang 2003, Shi & Massagué 2003).

BMP: bone morphogenetic protein

LTBP: latent TGF-β-binding protein

Smad: composite name from Sma (*Caenorhabditis elegans*) and Mad (*Drosophila melanogaster*)

R-Smad: receptor-activated Smad

MH: mad homology

ALK: activin receptor-like kinase

CBP: CREB (cAMP-responsive element-binding protein) binding protein

COMBINATORIAL RECEPTOR ASSEMBLY AND LIGAND BINDING SPECIFICITY

In humans, *Drosophila melanogaster*, and *Caenorhabditis elegans*, the number of TGF-β ligands greatly exceeds the number of type II and type I receptors. For example, the human genome encodes at least 29 and probably up to 42 TGF-β ligands that form homodimers and possibly heterodimers, whereas only five type II and seven type I receptors have been identified. Combinatorial interactions of type

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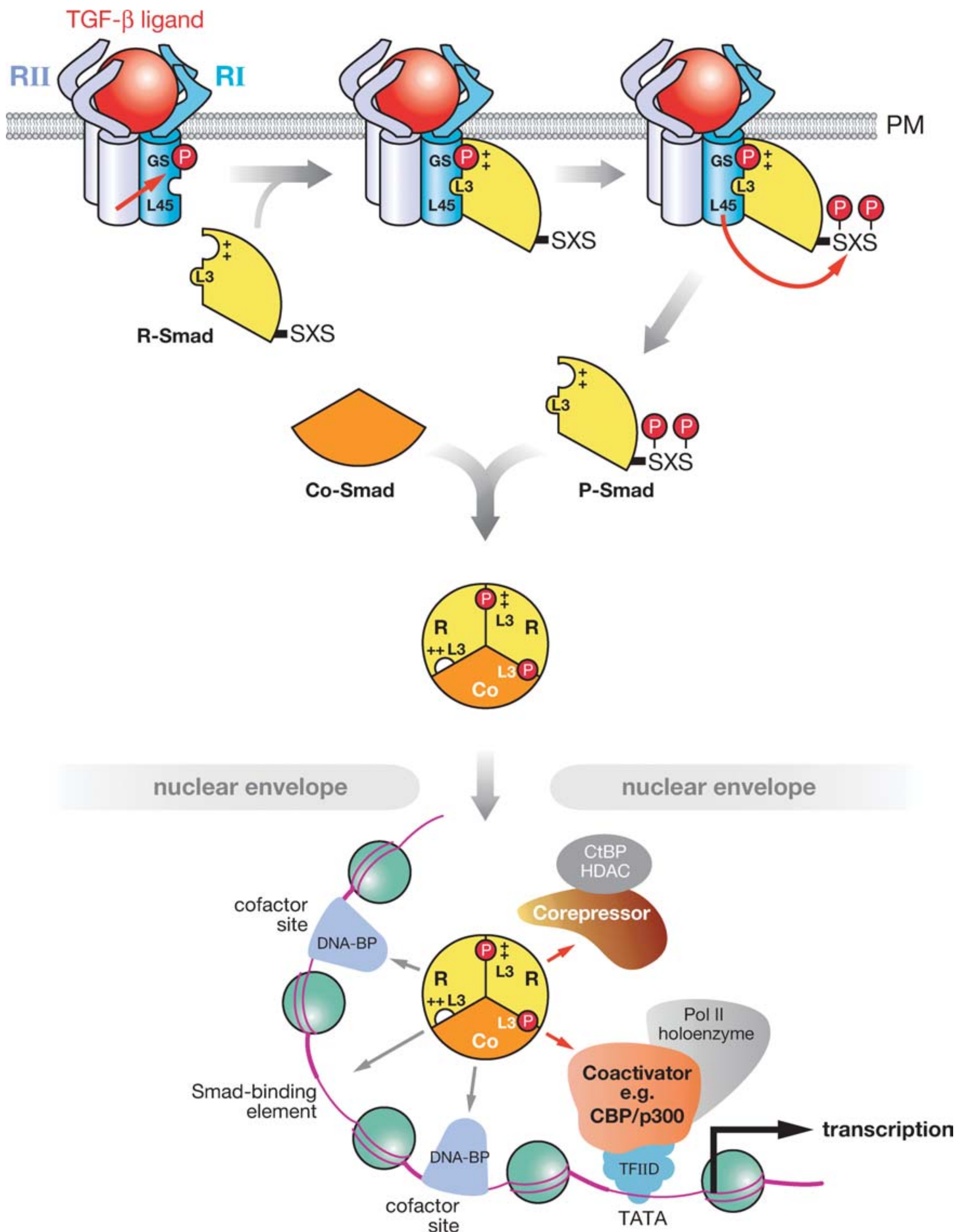

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I and type II receptors in functional receptor complexes allow for diversity and selectivity in ligand binding as well as in intracellular signaling.

The high number of ligands is best explained by the need for finely tuned developmental patterns of receptor activation, which is achieved in part by differential regulation of ligand expression and activation from latent complexes. Thus, even though multiple ligands may activate the same receptor complexes and signaling pathways, their distinct expression patterns set the stage for multiple and highly restricted roles of TGF-β family ligands using a small number of receptor combinations. The restricted patterns of receptor activation during development are further specified by the limited diffusion of TGF-β ligands and their association with divergent propolypeptides and LTBPs, which may specify selective activation mechanisms.

Type I and type II receptors exist as homodimers at the cell surface in the absence of ligands, yet have an inherent heteromeric affinity for each other. While one may theorize that all type II receptors could combine with all type I receptors, only select combinations act as ligand-binding signaling complexes (**Figure 2**). The molecular basis of the selectivity of the type II-type I receptor interactions is largely unknown, but the structural complement at the interface of the ligand-receptor interactions may help define the selectivity of the receptor combinations (Greenwald et al. 2004). Most ligands bind with high affinity to the type II or type I receptor, while others bind efficiently only to heteromeric receptor combinations. TGF-β1, TGF-β3 and activins bind efficiently to their respective type II receptors, TβRII and ActRII/ActRIIB, without the need for a type I receptor, yet the ligand contacts both receptor ectodomains to stabilize the type II-type I receptor complex (Boesen et al. 2002, Greenwald et al. 2004, Hart et al. 2002). In contrast, BMP-2 and -4 do not bind well to the type II receptor BMPRII, but bind efficiently to the type I receptors BMPRIA/ALK3 and BMPRIB/ALK6, and require the heteromeric complex for high affinity binding (Keller et al. 2004, Kirsch et al. 2000). Binding of TGF-β2 or BMP-7 requires both type II and type I receptor ectodomains (del Re et al. 2004, Greenwald et al. 2003). These and other observations provide evidence for the existence of unoccupied heteromeric receptor complexes at the cell surface.

In addition to binding of related ligands to the same receptor complex, a single ligand often activates several type II-type I receptor combinations. Dimeric TGF-β ligands have symmetric butterfly-like structures, whereby a monomer can be imagined as an open hand in which the central β-helix represents the wrist, the two aligned two-stranded β-sheets resemble four fingers, and the N-terminal sequence extends as a thumb (Shi & Massagué 2003). The BMP-2 homodimer complexed with two BMPRIA ectodomains shows two receptor-binding epitopes in the ligand that are conserved among BMPs (Keller et al. 2004, Kirsch et al. 2000). Superimposition of these data with the structure of BMP-7 in complex with ActRII ectodomains

Figure 1

The TGF-β signaling pathway. Ligands of the TGF-β superfamily first bind to the type II (RII) or type I (RI) homodimers or the RII-RI heterotetramer. Ligand binding stabilizes the receptor complex, in which RII phosphorylates the GS motif in the downstream type I receptor (RI) kinase. Following receptor activation, R-Smads are recruited to the receptor complex, primarily through an interaction between the L45 (on the RI) and L3 (on Smads) loops, and subsequently are phosphorylated in the SXS motif. Phosphorylated Smads (P-Smads) then form a trimeric complex with the common Smad4 in mammals. The Smad complex is then transported into the nucleus, where it interacts with DNA and transcription factors, including a large variety of DNA-binding transcription factors (DNA-BP) and coactivators or corepressors in a target gene–dependent manner.

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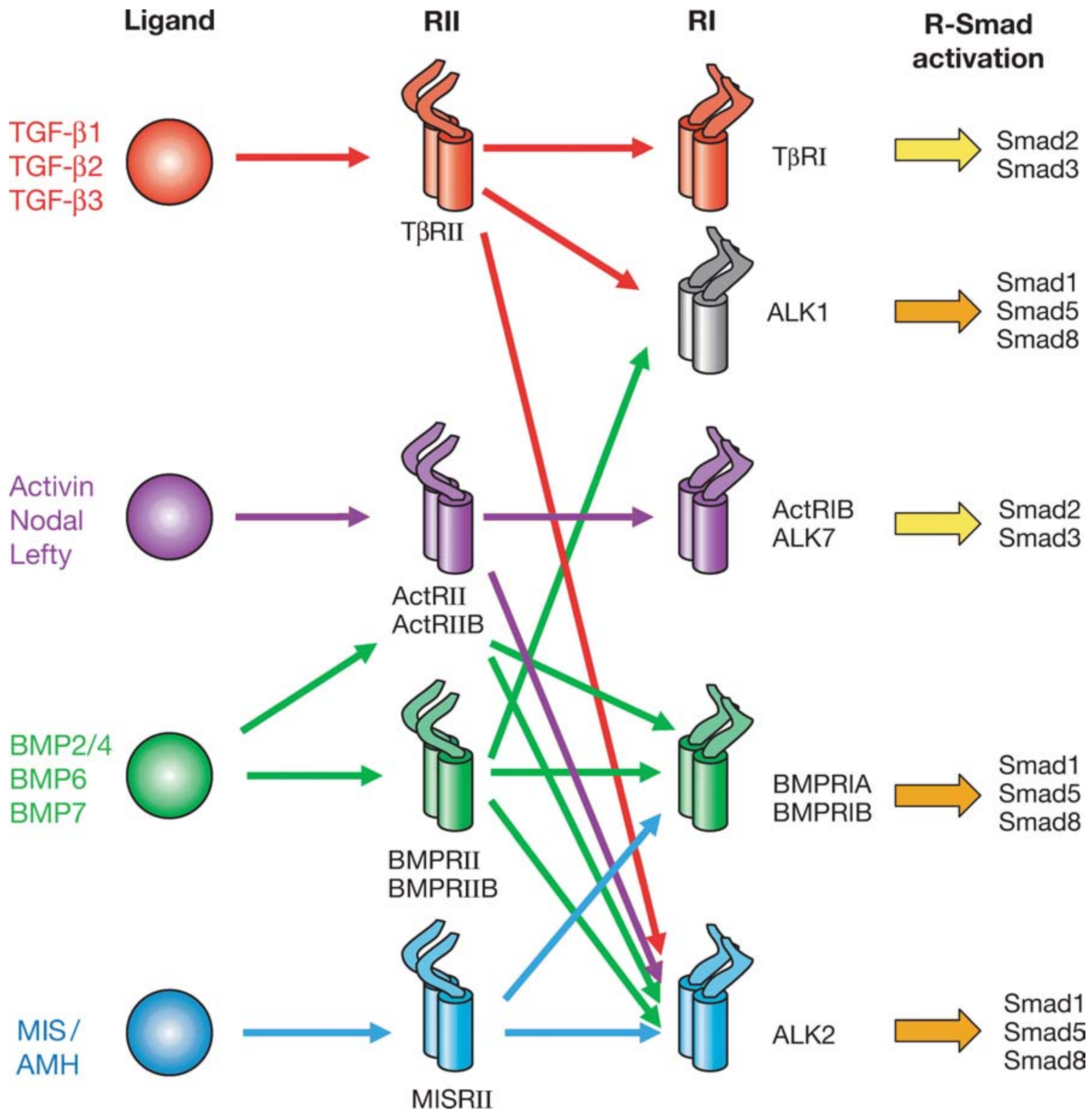

Figure 2

Heteromeric combination of TGF-β superfamily receptors. TGF-β ligands bind to specific combinations of RII-RI heterotetramers at the cell surface. Ligands, RII and RI are colorcoded for each pathway. Subsequent activation of R-Smads is shown at right. While ActRII and ActRIIB are encoded by different genes, BMPRII (long) and BMPRIIB (short) are two isoforms encoded by the same gene.

reveals that the type I and type II receptor extracellular domains in the tetrameric receptor complex do not interact with each other, yet allow cooperative ligand binding (Greenwald et al. 2004). This cooperativity in receptor binding may be modulated by the remarkable flexibility of the ligand (Sebald & Mueller 2003). The epitopes of TGF-β3 that bind TβRII are distinct from the receptor-binding epitopes in BMPs (Hart et al. 2002).

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Furthermore, in the complex of TGF-β3 with two TβRII and two TβRI ectodomains, the TβRII and TβRI domains not only contact the ligand but also interact with each other (Hart et al. 2002). The differences in these complexes, together with the flexibility in ligand binding to the receptor, provide a structural basis for the versatility of ligand binding to receptor complexes.

Since the signaling responses are defined by the composition of the receptor complex, in particular that of the type I receptor, a ligand can induce different responses, depending on the nature of the activated receptor complexes. For example, in addition to the well-characterized TβRII-TβRI complex, TβRII forms functional complexes with ActRI/ALK2 or ALK1; these complexes signal differently from those involving TβRI (Goumans et al. 2002). The opposing activities of TGF-β signaling through TβRI and ALK1, in complex with TβRII, define the balance in endothelial cell migration and proliferation (Goumans et al. 2003). Similarly, ActRIB/ALK4, in combination with ActRII or ActRIIB, activates activin-induced gene responses, and the ActRI-ActRII complex transduces BMP7 signals (Macias-Silva et al. 1998). In addition, BMP-RIA and BMP-RIB combine not only with the "classical" BMP-RII but also with ActRII (Macias-Silva et al. 1998, Nishitoh et al. 1996). Consistent with the picture of differential signaling responses, BMP-RIA is able to promote adipogenic differentiation, whereas BMP-RIB may be more potent in osteoblast differentiation (Chen et al. 1998a). Combinatorial use of receptors also occurs in *Drosophila*, in which the type I receptors Tkv and Sax interact with the type II receptor Punt to bind one of three ligands: Dpp, Gbb, and Screw. In addition to differential Smad activation, differences in ligand-induced internalization and routing of the receptor complex, depending on the ligand and receptor composition, are likely to define the signaling responses as well. Finally, heterodimeric ligands, such as inhibins or BMP heterodimers, may activate asymmetric receptor combinations with two different type II and/or type I receptors.

Accessory cell surface proteins further define the binding efficiency and specificity of the ligand to the receptor complex. Betaglycan and endoglin bind TGF-β with high affinity, yet have no known role as signaling effectors. Coexpression of betaglycan or endoglin enhances TGF-β responsiveness and TGF-β binding to the TβRII-TβRI complex. While betaglycan strikingly enhances the minimal binding of TGF-β2 to TβRII (López-Casillas et al. 1993), endoglin is required for efficient TGF-β signaling through TβRII-ALK1 in endothelial cells (Lebrin et al. 2004). Regulation of betaglycan expression at the cell surface by the PDZ protein GIPC may further define the response to TGF-β (Blobe et al. 2001). Furthermore, the cytoplasmic domain of betaglycan interacts with TβRII, which phosphorylates this domain. This further triggers the interaction of betaglycan with β-arrestin, thereby modulating the internalization of TGF-β receptor complexes (Chen et al. 2003).

Other coreceptors also act as determinants of ligand binding and signaling. For example, nodal acts through ActRIIB and ActRIB, but efficient binding and signaling by nodal requires interaction of Cripto or the related EGF-CFC proteins Cryptic or FRL-1 with ActRIB (Yeo & Whitman 2001). Cripto binds nodal via its EGF domain and ActRIB through its CFC domain (Yeo & Whitman 2001). Like TGF-β and nodal, BMPs also have a coreceptor called DRAGON, which directly interacts with ligands and receptors to facilitate BMP signaling (Samad et al. 2005).

SPECIFICITY AND COMPLEXITY IN SMAD ACTIVATION

Upon ligand binding, the activated type I receptors specify the gene expression responses. In the case of tyrosine kinase receptors, the activated signaling events are largely dictated by cytoplasmic sequences outside the kinase

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domains. In contrast, the type I receptors specify their signaling largely through the L45 loop sequence located within the kinase domain. The L45 loop serves as the key determinant in the recruitment of Smads and the specificity of signaling (Chen & Massagué 1999, Chen et al. 1998b, Feng & Derynck 1997). Once phosphorylated by type II receptors in response to ligand binding, the GS motif immediately preceding the kinase domain also contributes to the strength of Smad binding. The short juxtamembrane segment that precedes the GS motif does not seem to contribute to the specificity of Smad activation, even though this segment shows considerable sequence divergence. Smad binding to and phosphorylation by the type I receptor are further modulated by interacting proteins such as SARA and the inhibitory Smads.

Smad-Receptor Interactions

The specificity in gene expression responses is defined by differential Smad recruitment and activation by the type I receptors. As discussed above, the Smad-receptor interaction involves the L45 loop and the phosphorylated GS motif in the type I receptor. This interaction allows the type I receptor to phosphorylate the C-terminal SXS motif of the R-Smad, resulting in a conformational change in and dissociation of the activated Smad, with subsequent heteromerization with Smad4. Smad2 and 3 are phosphorylated by TβRI and ActRIB, whereas Smad1, Smad5, and Smad8 are substrates for BMP-RIA, BMP-RIB, ALK-1, and ALK-2 (**Figure 2**). The efficiency of Smad activation at the endogenous receptor and Smad levels, the quantitative dependence of Smad phosphorylation on ligand stimulation, and the relative affinities of the Smads for the receptors are likely to differ significantly among related receptors and Smads, but these quantitative assessments await characterization.

The nine-amino acid L45 loop, which connects β strands 4 and 5 in the kinase domains of the type I receptor, is accessible for protein interactions, as apparent from the structure of TβRI (Huse et al. 2001). Receptors with different signaling specificities have distinct L45 sequences (Chen et al. 1998b, Feng & Derynck 1997). The L45 sequences of TβRI and ActRIB, i.e., receptors that activate Smad2 and Smad3, are identical, but differ in four amino acids from the L45 sequences in BMP-RIA and -RIB, and in seven amino acids from the L45 loops of ALK-1 and ALK-2 (Chen et al. 1998b). Accordingly, replacement of the L45 sequence in ALK-2 with that of TβRI leads to TGF-β signaling (Feng & Derynck 1997). Similarly, TβRI with an L45 loop of BMP-RIB switches specificity to induce a BMP-like response, and a BMP-RIB with a TβRI L45 loop can activate TGF-β- and activin-like transcription, and does not activate BMP-inducible gene expression (Chen et al. 1998b).

The L45 loop interacts directly with the L3 loop in the MH2 domain of an R-Smad (Chen et al. 1998b, Lo et al. 1998). The L3 loop is located between two β sheets and is exposed in the trimeric Smad complexes (Shi & Massagué 2003). As with the L45 loop, only a few amino acids in the L3 loop define receptor-binding specificity. The L3 sequences are invariant between Smad2 and Smad3 as well as among BMP-activated Smad1, Smad5, and Smad8, but differ in two residues between both groups. Consequently, Smad1 with an L3 loop of Smad2 interacts with and is phosphorylated by TβRI, while Smad2 with the L3 loop of Smad1 no longer interacts with TGF-β receptors and is not phosphorylated by TβRI (Lo et al. 1998).

Adjacent sequences stabilize the interaction of the L3 and L45 loops and contribute to signaling specificity (Chen & Massagué 1999, Lagna & Hemmati-Brivanlou 1999, Lo et al. 1998). Smad1 with an L3 loop from Smad2 is still phosphorylated in response to BMP, albeit to a lesser extent than wild-type Smad1, and requires further replacement of the sequence downstream from the L3 loop with

that of Smad2 to abolish BMP-induced phosphorylation. Such larger replacement fully switches receptor binding specificity and confers efficient Smad phosphorylation by TβRI; this result, which could not be achieved by replacing the L3 loop alone (Chen & Massagué 1999), is due to the interaction of the phosphorylated GS motif with the Smad sequence downstream from the L3 loop. This notion is consistent with the current structural model for activation of the signaling response (Huse et al. 2001). The interaction of the L45 loop of a receptor with the L3 loop of an R-Smad may play an initial role in the receptor-Smad selection, but the interaction of the phosphorylated GS motif with the Smad sequence downstream from the L3 loop stabilizes the receptor-Smad interaction (Wu et al. 2001b).

Accessory Proteins in Smad Activation

Efficient R-Smad recruitment and activation in response to TGF-β or activin require SARA, an FYVE domain-containing protein that interacts with the type I receptor and Smad2/3 (Tsukazaki et al. 1998, Wu et al. 2000). SARA is localized at the plasma membrane and concentrated in EEA1-positive early endosomes through the interaction of the FYVE domain with the membrane lipid PtdIns(3)P. Complex formation of the receptors with SARA and Smad2/3 in early endosomes may thus be essential to efficiently initiate TGF-β signaling (Di Guglielmo et al. 2003, Hayes et al. 2002, Panopoulou et al. 2002). The structural interface of SARA and the interacting sequence in the MH2 domain of Smad2 reveal critical determinants of SARA-Smad interaction, explaining the inability of BMP-activated Smads to interact with SARA (Wu et al. 2000). This model also explains the dissociation of Smad2 from SARA, following Smad2's C-terminal phosphorylation by the type I receptor. Hgs, another FYVE domain protein involved in endosomal trafficking, may play a role similar to that of SARA, since it also interacts with Smad2 and Smad3 and enhances ligand-induced Smad phosphorylation and gene expression (Miura et al. 2000). However, the FYVE finger of SARA has a higher affinity for PtdIns(3)P than does the FYVE finger of Hgs, suggesting a predominant role for SARA in TGF-β and activin signaling (Panopoulou et al. 2002). Truncated versions of SARA or Hgs impair TGF-β/activin signaling, underscoring the roles of SARA and Hgs in activation of signaling (Miura et al. 2000, Panopoulou et al. 2002, Tsukazaki et al. 1998). Additional FYVE proteins yet to be identified may play similar roles in the binding selectivity and receptor interaction of the BMP-activated Smads1, 5, and 8.

Disabled-2 (Dab2), a protein that plays a role in multiple signaling pathways, also interacts with the TβRII-TβRI complex and Smad2/3. TGF-β enhances Dab2's interaction with these Smads and may stabilize the receptor-Smad interaction (Hocevar et al. 2001). The ability of Dab2 to interact with clathrin and the clathrin adaptor AP-2, and Dab2's localization to clathrin-coated pits, may link Dab2 to clathrin-mediated endocytosis of the activated TGF-β receptor complexes. Absence of Dab2 renders cells insensitive to TGF-β-induced Smad activation (Hocevar et al. 2001). Finally, Dok-1, a rasGAP-binding protein that acts downstream from receptor tyrosine kinases, is required for activin-induced Smad signaling. Dok-1 interacts with type II and type I activin receptors and activin triggers association of Dok-1 with Smad3 (Yamakawa et al. 2002).

Inhibitory Smad Interactions

Smad6 and Smad7, and presumably also Dad in *Drosophila*, inhibit TGF-β family signaling primarily by interfering with the receptor-mediated activation of R-Smads. These inhibitory Smads associate with type I receptors, thus competitively interfering with R-Smad

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recruitment and phosphorylation (Hayashi et al. 1997, Imamura et al. 1997, Nakao et al. 1997). Smads1 and 5 induce Smad6 expression, whereas Smad3 induces Smad7 expression. Consequently, BMP signaling induces an inhibitory feedback loop through Smad6 expression, while TGF-β induces an inhibitory feedback loop through Smad7 expression, although BMPs and TGF-β can also induce Smad7 and Smad6 expression, respectively. Smad6 inhibits BMP and TGF-β signaling with similar potency, while Smad7 inhibits TGF-β signaling more efficiently than Smad6 (Miyazono 2000). Through their MH2 domains, Smad6 and Smad7 interact with the type I receptors, and the isolated MH2 domains interact with similar affinity with TβRI (Hanyu et al. 2001, Souchelnytskyi et al. 1998). Presence of the MH1 domain of Smad7, but not that of Smad6, increases the interaction of the Smad7 MH2 domain with TβRI (Hanyu et al. 2001). Structural predictions combined with mutation analysis have identified a basic surface in the MH2 domain as critical for the interaction of Smad7 with TβRI. Two Lys residues are essential for the binding of Smad7 to TGF-β receptor complexes and inhibition of TGF-β signaling by Smad7, whereas two other basic residues in the L3 loop are essential for inhibiting both TGF-β and BMP signaling (Mochizuki et al. 2004). The WD-repeat protein STRAP-1, which interacts with the TGF-β receptors, assists in the interaction of Smad7 with these receptors and thus cooperates with Smad7 to inhibit TGF-β signaling (Datta & Moses 2000).

In addition to the competitive interference of Smad6 and Smad7 with R-Smad binding to type I receptors, Smad6 also inhibits complex formation of BMP-activated Smad1 with Smad4 (Hata et al. 1998). Smad6 and Smad7 also inhibit TGF-β family signaling by interacting directly with Smurf E3 ubiquitin ligases and mobilizing these ligases to the type I receptors, leading to proteasomal degradation of the receptors (Ebisawa et al. 2001, Kavsak et al. 2000, Murakami et al. 2003). Conversely, Smurf1 appears important in targeting Smad7 to the receptor complex (Suzuki et al. 2002).

Several signaling pathways lead to a rapid induction of Smad6 and Smad7 expression, which constitutes a critical point for negative regulation of TGF-β signaling. Most notably, TGF-β or BMP signaling induces Smad6 or Smad7 expression that can result in attenuation of ligand-induced Smad activation and gene expression. Smad7 is also induced by Jak/STAT signaling in response to interferon-γ (Ulloa et al. 1999) and by NF-κB signaling in response to inflammatory cytokines (e.g., TNF-α and IL-1) and lipopolysaccharide (Bitzer et al. 2000). In addition, fluid shear stress induces Smad6 and Smad7 expression in endothelial cells (Topper et al. 1997). The induced expression of inhibitory Smads consequently decreases receptor-mediated Smad activation and the cell's responsiveness to TGF-β ligands.

Heteromeric Smad Complex Formation

Upon release from the receptors, the phosphorylated R-Smads form complexes with Smad4 that act as effectors of ligand-induced signaling. Structural analyses have shown that the MH2 domains of Smad4 (Shi et al. 1997), pseudophosphorylated Smad3 (Chacko et al. 2001), and phosphorylated Smad2 form homotrimers (Wu et al. 2001b). In addition, the phosphorylated or pseudophosphorylated MH2 domains of Smad1 or Smad2/3 (Chacko et al. 2004, Qin et al. 2001, 2002) form heteromeric trimers with Smad4 consisting of two R-Smads and one Smad4. Smad homotrimerization and heterotrimerization in solution are also observed from biochemical analyses (Chacko et al. 2001, 2004, Jayaraman & Massagué 2000). High-affinity trimer formation is primarily mediated by the L3 loop in the MH2 domain and SXS phosphorylation (Chacko et al. 2001, 2004, Jayaraman & Massagué 2000). Thus, ligand-induced SXS phosphorylation of R-Smads may be a prerequisite for natural Smad trimerization.

This heterotrimeric Smad model is consistent with the requirement of Smad4 in most TGF-β-induced transcriptional responses. This structure also allows for combinatorial interactions and versatility and may explain the requirement of Smad2, Smad3, and Smad4 in the induction of transcription of the cdk inhibitors p15^{Ink4B} (Feng et al. 2000) and p21^{Cip1} (Pardali et al. 2000b). It is thus easily conceivable that two different BMP-activated Smads may combine with Smad4 or even that a BMP-activated Smad may combine with Smad2/3, and Smad4 as third partner, to activate or repress selective transcription responses. Competition of Smad3 with Smad2 for interaction with Smad4 may explain the ability of Smad3 to inhibit activin-induced goosecoid expression through Smad2/4 (Labbé et al. 1998).

The incorporation of Smad4, which has no SXS motif, into the activated R-Smad complex lends a possibility that Smad6 or Smad7 may form a complex with two R-Smads. This would be consistent with the observations that Smad6 interacts with Smad1 (Hata et al. 1998) and that Smad6 binding to the Id1 promoter requires the presence of Smad1 (Lin et al. 2003). Complex formation of Smad6 with R-Smads may provide a mechanism for functional repression of the effector functions of Smads. Replacement of Smad4 with Smad6 or Smad7 not only would eliminate Smad4 as coactivator, thus preventing gene activation, but would also recruit histone deacetylases to confer active gene repression. The coactivator role of Smad4 and possible roles of Smad6 and Smad7 in transcription repression will be discussed further below.

The evidence for Smad trimerization contrasts with some reports that Smads form dimers (Inman & Hill 2002, Jayaraman & Massagué 2000, Wu et al. 2001a). At promoter DNA, Smads may exist as trimers or dimers, depending on the interacting transcription factor (Inman & Hill 2002). Crystallographic analyses should provide insight into the characteristics of Smad complexes at DNA and their interactions with sequence-specific transcription factors.

Control of Smad Activation by Diverse Kinase Pathways

MAPK: mitogen-activated protein kinase

JNK: Jun N-terminal kinase

In addition to C-terminal SXS phosphorylation by type I receptors, R-Smad activation is regulated by cytoplasmic kinases. The linker regions of the R-Smads are targets for proline-directed kinases such as MAPKs and cyclin-dependent kinases. Erk MAPK, which is activated in response to mitogenic growth factors or oncogenic Ras mutants, can phosphorylate the linker regions of Smad1 and Smad2/3, thereby inhibiting ligand-induced nuclear translocation of Smads and consequently the TGF-β antiproliferative response (Kretzschmar et al. 1997, 1999, Pera et al. 2003). However, other studies did not observe impaired nuclear translocation of Smads in cells with activated Ras/MAPK signaling (de Caestecker et al. 1998, Engel et al. 1999). In addition, impaired Smad signaling in Ras transformed cells is not easily reconciled with the cooperation between Ras/MAPK and TGF-β signaling in tumor cell differentiation and behavior (Janda et al. 2002). Regulation of Smad activation by Erk MAPKs may also control developmental processes. In Xenopus embryos, FGF8, in combination with IGF2, induces a MAPK-dependent inhibitory phosphorylation of the Smad1 linker region, which contributes to the induction of a neural cell fate (Pera et al. 2003). It should be noted that TGF-β receptors can activate MAPK signaling (Derynck & Zhang 2003, Massagué 2003)

JNK, which is activated in response to mitogenic and stress signals, phosphorylates Smad3 outside its SXS motif and enhances activation and nuclear translocation of Smad3 (Engel et al. 1999). Furthermore, activation of MAPK kinase 1 (MEKK-1), an activator of JNK and Erk MAPK, leads to phosphorylation and activation of Smad2 (Brown et al. 1999). This mechanism may explain the ability of fluid shear stress or some growth factors to activate Smad2 (Brown et al. 1999,

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PKC: protein kinase C

CaMKII: calmodulin-dependent kinase II

SBE: Smad-binding element

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de Caestecker et al. 1998). This regulation can be further complemented by alterations in the stability of Smad4, e.g., through induction of proteolytic degradation in response to activated MAPK signaling (Liang et al. 2004, Saha et al. 2001).

The cyclin-dependent kinases CDK2 and CDK4 also phosphorylate the linker regions of Smads2 and 3, but at sites that differ from those targeted by Erk MAPK, and consequently inhibit Smad-dependent gene transcription and cell cycle arrest (Matsuura et al. 2004). Since tumor cells often activate these CDKs, inhibition of Smad activity by CDK-dependent phosphorylation may provide an escape from antiproliferative control by autocrine TGF-β signaling.

PKC and CaMKII also regulate Smad activation. PKC-dependent phosphorylation of the MH1 domain abolishes the DNA binding of Smad3 (Yakymovych et al. 2001). In mesangial cells, CaMKII phosphorylates Smad2, and to a lesser extent Smad3. One of the phosphoacceptor sites, Ser-240, which is in the linker region, is phosphorylated in response to EGF, PDGF, or TGF-β. CaMKII induces a Smad2-Smad4 complex independently of TGF-β receptor activation, but this complex may be inactive (Abdel-Wahab et al. 2002). Casein kinases I, which have been implicated in various processes, also control Smad activity. Casein kinase Iε associates with and can phosphorylate R-Smads, yet also interacts with TGF-β receptors. Consequently, casein kinase Iε may regulate TGF-β/Smad signaling (Waddell et al. 2004). Finally, Akt (protein kinase B), which can be activated in response to insulin, can associate directly with Smad2 and 3, and thus control their activation and the response to TGF-β. In response to insulin, Akt interacts with Smad3 that has not been phosphorylated in response to TGF-β. Consequently, Akt inhibits Smad3 activation by TGF-β and Smad3/4 complex formation and nuclear translocation, whereas TGF-β signaling decreases the partnering of Akt with Smad3. This balance results in the ability of Akt to decrease TGF-β/Smad3-mediated transcription and TGF-β-induced apoptosis (Conery et al. 2004, Remy et al. 2004).

Taken together, phosphorylation by MAPKs and kinases involved in other pathways exert differential effects by targeting distinct phosphorylation sites in the Smads, independently from C-terminal SXS phosphorylation by the type I receptor, but the outcome depends on the cell signaling context; depending on cell type and physiology, such phosphorylation even could exert opposite effects. The combination of these phosphorylation events greatly contribute to the final gene responses to Smad signaling (Massagué 2003).

SMADS IN THE NUCLEUS: SPECIFICITY AND VERSATILITY IN TRANSCRIPTIONAL CONTROL

Transcriptional activation by Smads is based on cooperation of the Smad complex with other DNA sequence-specific transcription factors at the promoter DNA. This interaction involves association of the Smad complex with the DNA-binding transcription factor, Smad binding to an adjacent DNA sequence and interaction of R-Smads with the CBP or p300 transcription coactivators. Smad4 then acts as Smad coactivator by stabilizing the interaction of activated R-Smads with CBP/p300. This mechanism allows for an extensive versatility, yet also confers specificity.

Smads as DNA-Binding Factors

Smads contact DNA selectively, with 5′-GTCTAGAC-3′ as the optimal sequence for Smad3 or Smad4 binding (Zawel et al. 1998). The Smad3 MH1 domain interacts through a β hairpin with the major groove of the DNA sequence 5′-GTCT-3′ and its reverse complement, 5′-CAGA-3′ (the SBE) (Shi et al. 1998). This interaction involves hydrogen bonds with the two G residues in the SBE. Since DNA binding of a Smad is marked

by minimal sequence requirements and low affinity, multiple Smad binding sites are required for Smad3-mediated transcriptional activation in the absence of an interacting, sequence-specific transcription factor. At natural promoters, however, a Smad binding sequence that is adjacent to the sequence binding the Smad-interacting transcription factor with high affinity allows binding of Smad transcription complexes. The juxtaposition of both sequences may result in an affinity exceeding that of the interacting transcription factor for its cognate DNA sequence, which may explain why Sp1 and c-Jun, when interacting with Smad3, bind their cognate DNA sequences with higher affinity than in the absence of Smad3 (Feng et al. 2000, Qing et al. 2000).

Smad3 also binds a GGCGGG sequence in the c-myc promoter; binding to this sequence, which was shown to bind E2F, is required for the transcriptional repression of c-myc by TGF-β signaling (Frederick et al. 2004). Although able to bind to the SBE sequence, Smad1 and its *Drosophila* homolog Mad bind to a GCCG sequence with higher affinity, which consequently confers BMP responsiveness (Kim et al. 1997, Korchynskyi & ten Dijke 2002, Kusanagi et al. 2000). Smad4 and its *Drosophila* homolog Medea also bind to GC-rich sequences (Ishida et al. 2000).

In contrast to other R-Smads such as Smad3, Smad2 is unable to bind DNA owing to a sequence insert in the β hairpin (Shi et al. 1998). However, a splicing variant of Smad2 with a deletion of this insert has similar DNA-binding properties as Smad3 (Yagi et al. 1999). It is thought that Smad2/4 complexes bind DNA through Smad4.

Combinatorial Interactions of Smads with DNA-Binding Transcription Factors

Smads cooperate through physical interactions with a remarkable diversity of DNA sequence–binding transcription factors (**Table 1**). These interactions occur through either the Smads' MH1 or MH2 domains, depending on the transcription factor. The regulation of the activities of the interacting transcription factors by other signaling pathways further defines this cooperation. This versatility explains the complexity and cell context dependence of the transcription programs exerted by TGF-β ligands, as well as why no consensus TGF-β ligand response sequences can be defined.

SIM: Smad interaction motif

FM: FoxH1 motif

FAST/FoxH1, a forkhead (Fox) transcription factor, was the first transcription factor reported to interact and cooperate with Smads in mediating TGF-β signals. In response to activin, Smad2/4 complexes interact with DNA-bound FoxH1 at an activin-response element and provide ligand-induced transcription (Chen et al. 1997). In this complex, Smad4 contacts the DNA while the MH2 domain of Smad2 interacts with FoxH1 (Labbé et al. 1998, Zhou et al. 1998). FoxH1 interacts with Smad2 using an SIM also present in Mix transcription factors and an FM uniquely present in FoxH1 (Randall et al. 2004). The SIM motif is also present in the Smad-binding domain of SARA, and is thus involved in the mutually exclusive Smad2-SARA and Smad2-FoxH1 interactions (Randall et al. 2002). Smad-FoxH1 cooperation mediates nodal signaling in endoderm and dorsal mesoderm formation in zebrafish (Sirotkin et al. 2000) and mice (Hoodless et al. 2001, Yamamoto et al. 2001). In mammals, FoxH1 cooperates with Nkx2.5 in Smad-dependent MEF2C expression, essential for heart looping morphogenesis (von Both et al. 2004).

Forkhead proteins also participate in the antiproliferative responses to TGF-β. In epithelial cells, TGF-β induces the expression of the CDK inhibitors $p21^{Cip1}$ and $p15^{Ink4B}$. At the $p21^{Cip1}$ promoter, the Smad3/4 complex interacts with FoxO, a target of the PI3 kinase/Akt pathway, to induce transcription of the $p21^{Cip1}$ gene (Seoane et al. 2004). FoxO binds to a distal sequence of the $p21^{Cip1}$ promoter, but Smads also interact with Sp1 at a proximal sequence to regulate $p21^{Cip1}$ expression (Pardali et al. 2000b), suggesting the

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Table 1 Smad-interacting DNA-binding transcription factors in mammalian cells

Smad-binding partners	Interacting Smad and domains	Features/mechanisms of action	References
bHLH family			
E2F4/5	Smad3 (MH2)	Recruitment of p107 to Smad3 to repress the c-myc gene	Chen et al. 2002
Max	Smad3 (MH1)	Max inhibits transcription activation by Smad3	Grinberg & Kerppola 2003
MyoD	Smad3 (MH1-linker)	Interference of MyoD/E protein/DNA complex formation	Liu et al. 2001
TFE3	Smad3/4	Synergistic cooperation on TGF-β target genes such as PAI-1, Smad7	Hua et al. 1999, Huse et al. 2001, Kawata et al. 2002
bZIP family			
ATF2	Smad3/4 (MH1)	Stimulation of ATF2 transactivation	Sano et al. 1999
ATF3	Smad3 (MH2)	Repression of the Id1 promoter	Kang et al. 2003
c-Fos	Smad3 (MH2)	Cooperation on AP-1-dependent TGF-β target genes	Zhang et al. 1998
c-Jun, JunB, JunD	Smad3 (MH1), Smad4	Positively and negatively regulate Smad activity	Liberati et al. 1999, Zhang et al. 1998
CEBPα, β, δ	Smad3 (MH1)	Smad3 inhibits CEBP's transactivation	Choy & Derynck 2003, Coyle-Rink et al. 2002
Forkhead family			
FoxH1/FAST	Smad2/3	Formation of activin-responsive factors on the activin-responsive promoters	Chen et al. 1997, Labbé et al. 1998, Randall et al. 2002
FoxO	Smad2/3	Regulation of $p21^{Cip1}$	Seoane et al. 2002
Homeodomain protein family			
Dlx1	Smad4	Inhibits Smad4 signaling	Chiba et al. 2003
Hoxc-8	Smad1 (MH1-linker), Smad6 (MH2)	Relief of Hoxc-8-dependent repression Inhibition of Smad1-Hoxc-8 interaction	Shi et al. 1999
Milk/Mixer	Smad2 (MH2)	Recruitment of Smad2/Smad4 activators to the activin-responsive complex	Germain et al. 2000, Randall et al. 2002
Nuclear receptor family			
Androgen receptor (AR)	Smad3 (MH2)	Reciprocal inhibition of Smad3 DNA-binding activity and of AR activity	Chipuk et al. 2002, Hayes et al. 2001, Kang et al. 2002
Estrogen receptor	Smad1/3/4(MH2)	Repression of Smad target genes	Matsuda et al. 2001, Wu et al. 2000, Zhang et al. 2000
Glucocorticoid receptor	Smad3 (MH2)	Inhibition of Smad3 transactivation activity	Song et al. 1999
HNF4	Smad3/4	Cooperative activation	Chou et al. 2003
RXR	Smad3 (MH2)		Pendaries et al. 2003
Vitamin D3 receptor	Smad3 (MH1)	Coactivation of ligand-induced transactivation of vitamin D receptor	Yanagisawa et al. 1999
Runx family			
CBFA1/Runx2/AML	Smad1/2/3/5 (MH2)	Cooperative activation of BMP responses; regulation of immune responses	Hanai et al. 1999, Pardali et al. 2000a, Zhang & Derynck 2000, Zhang et al. 2000

(*Continued*)

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Table 1 *(Continued)*

Smad-binding partners	Interacting Smad and domains	Features/mechanisms of action	References
Zinc finger protein family			
GATA3	Smad3	Recruits Smad3 to GATA sites to cooperatively activate transcription	Blokzijl et al. 2002
GATA4,5,6	Smad1	Cooperate in the regulation of Smad7 and Nkx2.5	Benchabane & Wrana 2003, Brown et al. 2004
GliΔC-ter	Smad1/2/3/4	Unknown	Liu et al. 1998
OAZ	Smad1/4 (MH2)	Formation of BMP-responsive activator complex	Hata et al. 2000
Sp1	Smad2 (MH1) Smad4 (MH2)	Cooperative activation of TGF-β target genes, e.g., p15^{Ink4B}, p21^{Cip1}, Smad7, PAI-1, and collagen	Feng et al. 2000, Pardali et al. 2000b
YY1	Smad1, Smad4 (MH1)	Complex with Smads and GATA	Kurisaki et al. 2003, Lee et al. 2004
ZNF198	Smad3 (MH2)	Unknown	Warner et al. 2003
Others			
β-catenin	Smad1/4	Wnt-dependent activation of LEF1 target genes	Hu et al. 2005, Hussein et al. 2003, Lei et al. 2004
HIF-1α	Smad3 (MH1, MH2)	Cooperation of TGF-β with hypoxia pathway and angiogenesis	Sanchez-Elsner et al. 2001
IRF-7 (IRFs)	Smad3 (MH2)	Smad3 activation of IRF-7 transactivation function	Qing et al. 2004
Lef1/TCF	Smad1/2/3/4 (MH1, MH2)	Smad coactivation of LEF1 signaling	Hu et al. 2005, Labbé et al. 2000, Nishita et al. 2000
MEF2 (MADS box)	Smad3	Smad3 represses the transcription activity of MEF2	Liu et al. 2004
Menin	Smad2/3 (MH2)	Facilitate Smad DNA binding	Kaji et al. 2001
NFκB p52	Smad3	Coactivation of κB site	Lopez-Rovira et al. 2000
NICD	Smad1/3 (MH2)	Coactivation of NICD-RBP-Jk complex to regulate the Notch targets	Blokzijl et al. 2003, Dahlqvist et al. 2003, Itoh et al. 2004, Zavadil et al. 2004
p53	Smad2/4	Synergism and antagonism	Chordenonsi et al. 2003, Takebayashi-Suzuki et al. 2003, Wilkinson et al. 2005
Pax8	Smad3	Smad3 reduces Pax8 DNA binding	Costamagna et al. 2003
SRF	Smad3	Mediate TGF-β-induced SM22α transcription	Qiu et al. 2003

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formation of multiple Smad complexes in a single promoter.

The interaction of Smads with Sp1 illustrates their cooperation with Zn finger transcription factors. Sp1, which uses the Mediator complex as a coactivator, drives transcription of the p15^{Ink4B} and p21^{Cip1} genes. At either promoter, TGF-β induces transcriptional cooperation of Smad2/3/4 complexes with Sp1 through association with a glutamine-rich domain in Sp1 (Feng et al. 2000, Pardali et al. 2000b). Smad-Sp1 interactions may also activate TGF-β-induced transcription of the α2(I) collagen, integrin β5,

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Smad7, and PAI-1 genes. No interactions of Smads with the related Sp2 and Sp3 transcription factors have been reported.

Additional Zn finger proteins participate in BMP or TGF-β signaling. Smad1 can associate with OAZ in activation of the Xvent2 gene (Hata et al. 2000). GATA transcription factors, which regulate cell differentiation, also interact with Smad proteins and modulate responses to BMP. Smad1 interactions with GATA4, 5, or 6 regulate transcription of the Smad7 (Benchabane & Wrana 2003) and Nkx2.5 (Brown et al. 2004) genes. At the Nkx2.5 promoter, this cooperation also involves another Zn finger protein named YY1. YY1 associates with Smad1/4 at adjacent YY1- and Smad-binding sites, thereby constituting a minimal BMP-responsive enhancer; thus, a multicomponent complex consisting of Smads, YY1, and GATAs regulates the BMP-responsiveness of the Nkx2.5 gene (Lee et al. 2004). YY1 and GATA proteins also mediate TGF-β responses. At the interleukin 5 promoter, TGF-β induces Smad3 recruitment to GATA3 at GATA-binding sequences independently of Smad3 binding to DNA, and functional cooperation of Smad3 with GATA3 to activate transcription (Blokzijl et al. 2002). Also, YY1 interaction with the MH1 domain of Smad4 or other Smads inhibits TGF-β-activated transcription (Kurisaki et al. 2003). In *Drosophila*, the Zn finger protein Schnurri is targeted by Dpp-activated Mad. Their interaction allows for transcriptional activation (Dai et al. 2000), yet suppresses transcription of Brinker, a repressor of Mad-mediated transcription (Marty et al. 2000). Finally, the Zn finger proteins Evi-1 (Kurokawa et al. 1998, Alliston et al. 2005) and SIP1 (Postigo et al. 2003, Verschueren et al. 1999) interact with Smads to repress Smad-mediated transcription, as will be discussed later.

Smads also interact with select bZIP family transcription factors, which contain basic and leucine zipper domains involved in DNA binding and dimerization. Among these, Smad3 can interact with c-Jun, JunB, ATF-2, ATF3, and, with lower efficiency, c-Fos in response to TGF-β (Kang et al. 2003, Liberati et al. 1999, Sano et al. 1999, Zhang et al. 1998). c-Jun, JunB, and ATF-2 interact through their bZIP domains with the MH1 domain of Smad3, while c-Fos and ATF3 interact with the MH2 domain of Smad3. While the stoichiometry and configuration of these interactions at the DNA are unclear, the enhanced transcription presumably results from cooperative recruitment of CBP/p300. Smad3 and CREB similarly can cooperate at adjacent DNA sequences, even though no physical interaction is detected (Zhang & Derynck 2000). Since AP-1 complexes of c-Jun and c-Fos, or related dimers, mediate responses to mitogenic factors and stress, the cooperation of Smads with bZIP transcription complexes at TGF-β-responsive promoters provides a mechanism for convergence of both signaling pathways. Smad3 and Smad4 also associate with the C/EBP transcription factors. Interaction of Smad 3/4 with C/EBPβ mediates the TGF-β-dependent inhibition of adipocyte differentiation (Choy & Derynck 2003) and HIV Tat–mediated transcription (Coyle-Rink et al. 2002).

Several homeodomain transcription factors, which play crucial roles in patterning and tissue differentiation, are also targeted by Smad signaling. In *Drosophila*, Medea cooperates with Tinman, the homolog of Nkx2.5, to induce tinman transcription in response to Dpp. In Xenopus, Smad2/4 interacts with Mixer and Milk to activate activin-responsive transcription of the goosecoid gene (Germain et al. 2000). As in the case of FoxH1, these Mix proteins interact through their SIM sequences with the MH2 domain of Smad2 (Randall et al. 2002). Homeoproteins may also oppose TGF-β signaling. For example, Dlx1 interacts with Smad4 and blocks signals from TGF-β proteins in hematopoiesis and perhaps neurogenesis (Chiba et al. 2003). At the osteopontin promoter, Smad1/4 interacts with Hoxc-8 and blocks Hoxc-8 binding to the homeodomain-binding sequence, thereby

preventing Hoxc-8-mediated transcriptional repression and allowing transcription in response to BMP (Shi et al. 1999).

In response to TGF-β, Smad3 can interact and cooperate with some bHLH transcription factors, which are characterized by a basic helix-loop-helix domain involved in DNA binding and dimerization. Smad3 cooperates with TFE3 in the transcription of the plasminogen activator inhibitor-1 (Hua et al. 1999), Smad7 (Hua et al. 2000), and laminin γ-chain (Kawata et al. 2002) genes. Smad3 also interacts with the myogenic bHLH transcription factors MyoD and myogenin (Liu et al. 2001), E2F4 (Chen et al. 2002), c-Myc (Feng et al. 2002), and Max (Grinberg & Kerppola 2003), but these interactions result in transcription repression, as will be discussed.

Smad3 also cooperates with Runt transcription factors. Runt proteins have a domain with homology to *Drosophila* Runt that interacts with DNA and promotes dimerization with a β subunit. Runx1/AML1 and Runx3/AML2 bind the germ line IgCα gene promoter at sequences adjacent to SBEs and cooperate with Smad3/4 to induce transcription in response to TGF-β, leading to IgA class switching (Hanai et al. 1999, Pardali et al. 2000a, Zhang & Derynck 2000). Smad3/4 also cooperates with Runx2/CBFA1 to induce transcription (Zhang et al. 2000), but this cooperation leads to repression of the Runx2 activity at the runx2 and osteocalcin promoters in mesenchymal cells (Alliston et al. 2001).

Smad3 also associates and cooperates with IRF-7, a member of the IRF transcription factors, which are involved in responses to viral and bacterial infection and inflammation. Smad3 cooperates with IRF-7 in the expression of interferon-β in response to polyI:C (Qing et al. 2004) through interaction of the MH2 domain of Smad3 with the transactivation domain of IRF-7. The transactivation domain of IRF-3, which resembles that of IRF-7, has a structure remarkably similar to the MH2 transactivation domain of Smads (Qin et al. 2003), raising the possibility that a heteromeric Smad-IRF complex may reciprocally regulate the transcription of Smad and IRF target genes.

IRF: interferon regulatory factor

Several intracellular receptors are targeted by TGF-β-activated Smad3 for functional cooperativity. The interaction of Smad3 with the vitamin D3 receptor (Yanagisawa et al. 1999) or HNF-4 (Chou et al. 2003) can result in transcriptional activation, while the glucocorticoid (Song et al. 1999), estrogen (Matsuda et al. 2001), and retinoic acid receptors (Pendaries et al. 2003) can repress the transactivation function of Smad3. Smad3 also interacts with the androgen receptor (Chipuk et al. 2002, Hayes et al. 2001, Kang et al. 2002). Other Smads can crosstalk with nuclear receptors as well. Smad4 binds to estrogen receptor α and represses estrogen gene responses (Wu et al. 2003). Estrogen induces an interaction between the estrogen receptor and Smad1 to inhibit Smad activity (Yamamoto et al. 2002). The functional consequences of many of these interactions require further characterization.

The cooperation of activin and Wnt signaling in tissue differentiation can result from interactions of Smad signaling with Wnt signaling effectors. Wnt signaling is mediated by the HMG box domain transcription factors LEF1 or TCF and their coactivator β-catenin. Smad3 and Smad4 can associate and cooperate with LEF1/TCF at the Xenopus twin promoter (Labbé et al. 2000, Nishita et al. 2000). At the myc promoter, which contains Smad- and TCF-binding sites, BMP can induce interaction of Smad1 with β-catenin and TCF4 to stimulate myc transcription (Hu & Rosenblum 2005). Similar crosstalk of both pathways is likely to regulate other developmentally regulated genes (Hussein et al. 2003, Lei et al. 2004). Smad3 also interacts with axin, a negative regulator of Wnt signaling with which several Wnt signaling mediators interact. TGF-β induces dissociation of a Smad3/axin complex and axin enhances TGF-β/Smad3 signaling, suggesting a role for axin in TGF-β signaling (Furuhashi et al. 2001).

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NICD: Notch intracellular domain

Like TGF-β and Wnt signaling, the Notch pathway controls cell differentiation. Activation of transmembrane Notch induces cytosolic release of its intracellular domain (NICD), which enters the nucleus where it interacts with the DNA-binding factor CSL/RBP-Jκ and activates Notch target genes repressed by CSL in the absence of Notch. TGF-β and BMP regulate Notch target gene expression through, respectively, the interaction of TGF-β-activated Smad3 and BMP-activated Smad1 with NICD (Blokzijl et al. 2003, Dahlqvist et al. 2003, Itoh et al. 2004, Zavadil et al. 2004). The Smad1-NICD interaction is further stabilized by associations with the p300/CBP and P/CAF coactivators (Itoh et al. 2004). This crosstalk leads to transcriptional cooperation or antagonism, depending on the gene and cell context. In myogenesis, upregulation of Hes and Hey1 expression by Notch signaling is required for TGF-β/BMP-mediated inhibition of differentiation (Blokzijl et al. 2003, Dahlqvist et al. 2003), whereas in endothelial cells, Herp2 expression in response to Notch inhibits cell migration by antagonizing BMP-induced Id1 function (Itoh et al. 2004).

TGF-β/Smad signaling also crosstalks with NF-κB signaling. NF-κB acts as a DNA-binding homodimer or heterodimer to induce transcription in response to inflammatory stimuli. TGF-β signaling can cooperate with NF-κB transcription through interaction of Smad3 with the p52 NF-κB subunit at adjacent NF-κB and Smad binding sites (Lopez-Rovira et al. 2000). Since NF-κB and R-Smads both interact with CBP/p300, their cooperation is likely a result of coordinately increased recruitment of CBP/p300, similar to the cooperation of Smad3 with many sequence-specific transcription factors.

Finally, TGF-β family signaling also synergizes with the p53 tumor suppressor, a regulator of cell proliferation, apoptosis, and differentiation. TGF-β/BMP signaling results in the formation of a p53-Smad complex that activates the transcription of target genes with distinct p53- and Smad-binding DNA sequences in their promoters. (Cordenonsi et al. 2003, Takebayashi-Suzuki et al. 2003). Furthermore, TGF-β treatment recruits p53, Smad2/4, and SnoN to adjacent SBE- and p53-binding sequences in the α-fetoprotein gene regulatory sequences, leading to transcription repression (Wilkinson et al. 2005).

In summary, the cooperation of Smads with DNA-binding transcription factors creates extensive versatility in the transcriptional regulation of target genes. Activated transcription often results from the interaction of the activated Smad complex with one DNA-binding transcription factor, but a higher level of complexity in which the Smad complex interacts with one or several DNA-binding transcription factors can occur, depending on the physiological context. This more complex scenario of transcriptional control with multiple Smad complexes or a larger complex may play out in the regulation of Smad7 transcription, through interactions of Smads with TFE3, AP-1, and Sp1 (Brodin et al. 2000, Hua et al. 2000); or the germ line IgCα promoter, through interactions of Smads with CREB and Runx proteins (Zhang & Derynck 2000). Such complex regulation may involve several Smad-binding sequences in addition to the DNA-binding sites for Smad-interacting transcription factors as in the promoter regions of the IgCα (Zhang & Derynck 2000), p15^{Ink4B} (Feng et al. 2000, Seoane et al. 2001), and p21^{Cip1} genes (Pardali et al. 2000b, Seoane et al. 2002).

Coactivators and Corepressors of Smads

In addition to interactions with DNA-binding transcription factors, Smads can recruit coactivators or corepressors into the transcription machinery that determine the amplitude of TGF-β/Smad-mediated transcriptional activation (**Table 2**).

Transcription coactivators, such as CBP/p300 and the Mediator complex, increase transcription by bringing the sequence-specific transcription factors into proximity

Table 2 Transcriptional coactivators and corepressors for Smads

Cofactors	Smad and Domains	Function	Reference
Coactivators			
ARC105	Smad2/3/4 (MH2)	Component of the ARC/Mediator	Kato et al. 2002
CBP/p300	Smad1/2/3 (MH2) Smad3 (linker) Smad4 (SAD)	Modulate chromatin structure and bridging TGF-β-independent transactivation function Smads with basic transcription machinery	Feng et al. 1998 Wang et al. 2005 de Caestecker et al. 2000
GCN5	Smad1/2/3/5	Modulation of chromatin structure	Kahata et al. 2004
MSG1	Smad4 (MH2)	Activation of CBP/p300-dependent transcription	Shioda et al. 1998
PCAF	Smad2/3 (MH2)	Modulation of chromatin structure and stimulation of CBP/p300-dependent transcription	Itoh et al. 2004
SKIP	Smad2/3 (Linker-MH2)	Derepression of Ski/SnoN?	Leong et al. 2001
SMIF	Smad4	Enhanced Smad4 coactivator function	Bai & Cao 2002
Swift	Smad2	Enhanced Smad2 transactivation function	Shimizu et al. 2001
ZEB1	Smad1/2/3/5 (MH2)	Promotes the formation of a p300-Smad transcriptional complex	Postigo 2003, Postigo et al. 2003
Corepressors			
c-Myc	Smad2/3 (MH2)	Inhibition of Smad-Sp1 activator complex	Feng et al. 2002
c-Ski, SnoN	Smad2/3/4 (MH2)	Recruits N-CoR, mSin3 and HADC	Luo et al. 1999, Wang et al. 2000, Wu et al. 2002
Evi-1 (ZF)	Smad1/2/3/4 (MH2)	Evi-1 is a zinc finger protein and recruits CtBP to repression complex	Izutsu et al. 2001, Kurokawa et al. 1998
SNIP1 (FHA)	Smad1/2/4	Inhibition of Smad4-p300 complex formation	Kim et al. 2000
TGIF (HD)	Smad2 (MH2)	Recruits CtBP and HDAC	Wotton & Massague 2001 and references therein
Tob	Smad1/5/8/4 Smad2/4	Targeting of BMP R-Smad to nuclear body Enhancement of Smad4 DNA-binding	Yoshida et al 2000 Tzachanis et al. 2001
YB-1	Smad3	Disrupt Smad3-DNA and Smad3-p300 interactions	Higashi et al. 2003
ZEB2/SIP1	Smad1/2/3/5 (MH2)	Recruitment of CtBP	Postigo 2003, Postigo et al. 2003, Verschueren et al. 1999

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to the RNA polymerase II complex. Some coactivators, e.g., CBP and p300, possess histone acetyltransferase (HAT) activity to modify chromatin structure. Through their MH2 domains, R-Smads directly interact with CBP or p300; their efficient interaction requires C-terminal SXS phosphorylation. This interaction is required for the transactivation function of the MH2 domain. The ligand-independent interactions of CBP/p300 with the linker region of Smad3, and possibly those of other Smads, contribute to full Smad3 activity (Wang et al. 2005).

The function of CBP as an R-Smad coactivator requires Smad4, which stabilizes the R-Smad interaction with CBP (Feng et al. 1998). The MH2 domain of Smad4 does not associate with CBP/p300 and has no transcription activity. However, inclusion of a proline-rich "SAD domain" upstream of the MH2 domain confers Smad4-dependent transcription (de Caestecker et al. 2000). This domain

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interacts with an N-terminal segment of p300 (de Caestecker et al. 2000) and also recruits SMIF, which has intrinsic transcription activity (Bai et al. 2002). Thus, a mutant Smad4 that does not interact with CBP/p300 yet retains SMIF binding is transcriptionally active (Bai et al. 2002). The interaction of SMIF with Smad4 suggests a function for SMIF in signaling by all TGF-β family members, irrespective of the nature of the activated R-Smad.

The coactivator functions of Smad4 and CBP/p300, and Smad-mediated transcription, can be further enhanced by MSG1. This coactivator interacts through a C-terminal domain with p300/CBP, and its N-terminal domain with the MH2 domain of Smad4 (Shioda et al. 1998). ZEB1, a Zn finger protein similar to ZEB1/SIP1, also enhances TGF-β signaling by promoting Smad3-p300/CBP interaction (Postigo et al. 2003).

The p300/CBP-associated PCAF and GCN5, two related coactivators, associate with Smad2 and Smad3 and potentiate TGF-β-induced transcription responses (Itoh et al. 2000, Kahata et al. 2004). GCN5, but not PCAF, also interacts with BMP-activated R-Smads and enhances BMP signaling (Kahata et al. 2004). Whether PCAF and GCN5 enhance Smad signaling through their ability to modify histones remains to be shown.

The ARC or Mediator complex acts as a coactivator in transcription through its interaction with RNA polymerase II, and may be a target of diverse regulatory circuits. ARC105, a component of this complex, is recruited to the Smad-responsive promoter in response to activin/nodal and binds Smad2/3 and Smad4, but not Smad1, in response to TGF-β (Kato et al. 2002). Thus, the Smad-ARC105 interaction mediates and relays TGF-β signaling to the Pol II machinery, which activates select genes. It is possible that BMP signals impinge on a distinct ARC component that interacts with a BMP-activated R-Smad and helps control BMP-responsive transcription.

Finally, the coactivator *Swift* interacts with Smad2 and has intrinsic transcription activity. Although it also interacts with Smad1, Swift enhances only activin/Smad2-mediated transcription and not BMP-induced responses in Xenopus embryos (Shimizu et al. 2001).

Corepressors that directly interact with Smads repress transcription induced by Smads. Several proto-oncogenes, including c-Ski/SnoN, c-Myc, and Evi-1, link repression of TGF-β/Smad signaling to malignant transformation. For example, c-Ski interacts with the MH2 domains of Smad2 and Smad3; increased expression of c-Ski or the related SnoN decreases activation of transcription by Smads (Luo 2004). In response to TGF-β, c-Ski inhibits both the induction of $p15^{Ink4B}$ and the downregulation of c-Myc expression, and consequently abolishes the growth inhibitory functions of TGF-β (Sun et al. 1999). c-Ski represses not only Smad2/3 responses but also BMP signaling through interaction with BMP-activated Smads and Smad4 (Wang et al. 2000). Additionally, c-Ski disrupts the functional complex of R-Smads with Smad4 (Wu et al. 2002), and recruits the nuclear N-CoR or mSin3 corepressors and interacting histone deacetylase(s) into the transcription complex (Luo et al. 1999), thus providing a dual mechanism of repression. The nuclear hormone receptor coactivator SKIP (Ski-interacting protein), which also interacts with the MH2 domain of Smad2 or Smad3, opposes the c-Ski-dependent repression of Smad transactivation and thus enhances Smad-mediated TGF-β responses (Leong et al. 2001).

c-Myc represses expression of $p15^{Ink4B}$ and $p21^{Cip1}$. At the $p15^{Ink4B}$ promoter, c-Myc associates with Smad2 and Smad3 and does not interfere with the formation of the Smad-Sp1 activator complex (Feng et al. 2002). The interaction of c-Myc with Sp1 presumably helps stabilize the interaction of c-Myc with the Smad-Sp1 complex and represses the functional cooperation between the Smad complex and Sp1 (Feng et al. 2002). c-Myc also interacts with the Zn finger protein Miz-1 near the transcription initiation site of the $p15^{Ink4B}$ promoter and thereby represses the ability of Miz-1 to activate $p15^{Ink4B}$ expression.

Repression of c-myc expression in response to TGF-β results in decreased interaction of c-Myc with Miz-1 (Seoane et al. 2001), thus conferring derepression that allows for Smad/Sp1-mediated transcription activation.

Evi-1, a Zn finger transcription factor, also represses Smad-mediated signaling. The repression of growth inhibition by TGF-β is likely the basis of the oncogenic function of Evi-1. Evi-1 interacts with the MH2 domain of Smad3 and other R-Smads, and thereby represses their transactivation function (Kurokawa et al. 1998, Alliston et al. 2005). Consequently, Evi-1 represses gene expression that is activated by activin, TGF-β, and BMPs (Alliston et al. 2005). The repressor activity of Evi-1 requires direct association with the corepressor CtBP (Alliston et al. 2005, Izutsu et al. 2001).

The homeobox transcription factor TGIF can also interact with Smads to repress Smad-mediated transcription. TGIF recruits histone deacetylases through its interaction with mSin3 and CtBP and competes with CBP/p300 for the R-Smad interaction (Wotton & Massagué 2001). Thus, TGIF acts through histone deacetylase–dependent and –independent mechanisms to repress TGF-β/Smad-activated transcription. The corepressor activity of TGIF is not restricted to TGF-β/Smad signaling, since TGIF binds cognate DNA sequences via its homeodomain and thus represses transcription independently of its interactions with Smads (Wotton & Massagué 2001).

A similar mechanism may account for the corepressor function of ZEB2/SIP1, a Zn finger/homeodomain protein that binds E-box sequences. SIP1 interacts with the MH2 domains of Smads (Postigo 2003, Verschueren et al. 1999) and represses Smad-mediated transcription depending on a DNA sequence that allows SIP1 binding (Comijn et al. 2001). Interestingly, the related ZEB-1/δEF1 protein, which also binds E-box sequences and can interact with Smad MH2 domains, activates TGF-β/BMP signaling (Postigo 2003). SIP1 downregulates hTERT (Lin & Elledge 2003) and E-cadherin expression (Comijn et al. 2001). Therefore, TGF-β-induced SIP1 expression can contribute to TGF-β-induced epithelial-to-mesenchymal transdifferentiation (Comijn et al. 2001) and inhibition of cellular transformation (Lin & Elledge 2003).

SNIP1 is yet another nuclear protein that can repress Smad-activated transcription. SNIP1 can interact with R-Smads and Smad4 as well as CBP/p300 (Kim et al. 2000). Thus, SNIP1 represses not only Smad-mediated transcription but also other responses that use CBP/p300 as coactivators.

Finally, Tob, a member of the Tob/BTG family of proteins with antiproliferative activities, participates in the regulation of both TGF-β and BMP signaling. Tob interacts with BMP-activated Smads and inhibits the stimulatory effect of BMPs on osteoblast function and bone deposition (Yoshida et al. 2000). In TGF-β signaling, interaction of Tob with Smad2 represses expression of interleukin-2 in T cells (Tzachanis et al. 2001). Tob proteins also bind inhibitory Smads and enhance their interactions with receptors, thereby inhibiting TGF-β signaling at the receptor level (Yoshida et al. 2003). Tob's mechanism of repression remains to be characterized.

Transcriptional Activation Versus Repression

Compared to Smad-mediated transcriptional activation, much less is known about the mechanisms of transcriptional repression by TGF-β family factors. Downregulation of c-myc expression has a key role in the growth inhibition response to TGF-β; preventing c-myc downregulation confers resistance to growth inhibition by TGF-β (Chen et al. 2001). The c-myc promoter contains a sequence that resembles the TIE in the promoter of the stromelysin 1 gene, which is also downregulated in response to TGF-β (Chen et al. 2002, Yagi et al. 2002). The c-myc TIE binds Smad3/4, E2F-4, and p107, and confers

TIE: TGF-β inhibitory element

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a transcriptional repression response to TGF-β (Chen et al. 2002, Frederick et al. 2004). The sequence that binds Smad3 is distinct from an SBE and overlaps with a consensus E2F site that binds E2F4/5 and recruits p107 (Frederick et al. 2004). First, Smad3 forms a complex with E2F4/DP-1/p107 in the cytoplasm, and in response to TGF-β the complex associates with Smad4 and occupies the TIE. It is not clear whether or how Smad3 and E2F4 simultaneously bind to the TIE sequence to repress the c-myc promoter (Chen et al. 2002, Frederick et al. 2004).

The inhibition of osteoblast differentiation by TGF-β is mediated in part by the interaction of Smad3 with Runx2, leading to repression of Runx2 transcription activity. TGF-β/Smad3-mediated repression of Runx2 neither requires DNA binding of Smad3 to the promoter nor results from decreased Runx2 binding to its cognate DNA sequence (Alliston et al. 2001). On the basis of a comparison of the responses at the Runx2-binding sequences in the osteocalcin and IgCα promoter sequences, it is apparent that the DNA sequence and cell type are important determinants. Indeed, depending on the DNA sequence, Smad3 cooperates with Runx2 to enhance or repress transcription. In addition, at the Runx2 binding sequence of the osteocalcin promoter, TGF-β and Smad3 repress Runx2-mediated transcription in mesenchymal cells but enhance it in epithelial cells (Alliston et al. 2001). Thus, cell type–dependent factors are key determinants of Smad-dependent activation versus repression. In osteoblasts and other mesenchymal cells, this repression of Runx2 by Smad3 is mediated by the direct recruitment of class IIa histone deacetylases, specifically HDAC4 and HDAC5, by TGF-β-activated Smad3 to the Runx2-binding DNA sequence in the osteocalcin promoter, thus resulting in histone deacetylation (Kang et al. 2005).

Recruitment of histone deacetylases has also been invoked in TGF-β family–induced transcription repression. BMP signaling results in the formation of a complex of Nkx3.2, HDAC1, and Smad1, and represses the transcription activity of Nkx3.2. The interaction of Nkx3.2 with HDAC/Sin3A requires the interaction of Nkx3.2 with Smad1 and Smad4. Thus, as in the case of TGF-β, BMP-activated Smads support ligand-induced transcription repression (Kim & Lassar 2003).

A different mechanism of Smad-mediated repression operates in the inhibition of myogenic differentiation by TGF-β. In response to TGF-β, Smad3 represses the activity of MyoD and myogenin through its direct interaction with the HLH domains of MyoD or myogenin (Liu et al. 2001). As a consequence, Smad3 interferes with the heterodimerization of MyoD or myogenin with their obligatory partner E12/47, thereby decreasing the DNA binding of MyoD or myogenin. Smad3 also interacts with MEF2C, which is a direct DNA-binding transcription factor and also serves as a coactivator required for efficient transcription by myogenic bHLH transcription factors. This interaction of Smad3 prevents MEF2C from associating with the MyoD/E protein complex and GRIP1, a coactivator that is required for the transcription functions of MEF2C (Liu et al. 2004).

Finally, Smad3/4 repress C/EBPβ- and STAT-3-mediated transcription of the haptoglobin promoter (Zauberman et al. 2001) and Smad3 represses the transactivation functions of C/EBPs, leading to transcriptional repression of the PPAR-γ promoter (Choy & Derynck 2003). The mechanisms for these cases of repression have not been characterized.

Taken together, the mechanistic differences of Smad-mediated repression versus activation remain to be fully characterized, yet are determined by cell type– and DNA sequence–dependent factors. In some cases, histone deacetylase–independent mechanisms mediate Smad-dependent repression, as in the repression of myogenic bHLH transcription factors and MEF2 (Liu et al. 2001, 2004). In other cases, e.g., the repression of Runx2 and Nkx3.2, histone deacetylase recruitment is involved. The

interaction of Smad3 with HDAC4 and HDAC5 (Kang et al. 2005) or with a different histone deacetylase activity (Liberati et al. 2001) illustrates the function of Smads as transcription repressors.

Inhibitory Smads as Transcription Regulators

Although inhibitory Smads interfere with receptor-mediated activation of R-Smads, several lines of evidence indicate that Smad6 and 7 also act as transcription regulators in the nucleus. Smad6 can physically interact with the corepressor CtBP; this interaction is mediated by Smad6's PLDLS motif, which is found in many repressors and confers intrinsic repressor activity to Smad6. Smad6-CtBP complexes are found at the BMP-responsive Id1 promoter and repress Id1 transcription (Lin et al. 2003). Smad6 can also interact with homeobox transcription factors at the DNA and thereby functions as corepressor (Bai et al. 2000). These interactions may also recruit class I histone deacetylases such as HDAC1 to repress BMP-induced gene transcription (Bai & Cao 2002). Although Smad7 does not interact with CtBP (Lin et al. 2003), it may also possess intrinsic transcription functions. Like Smad6, Smad7 is primarily localized in the nucleus. When fused to the DNA-binding domain of the Gal4 transcription factor, Smad7 can transactivate a Gal4 reporter gene (Pulaski et al. 2001). Furthermore, Smad7 interacts with and can be acetylated by the coactivator p300, further implicating a possible function of Smad7 in the nucleus (Grönroos et al. 2002).

CONCLUSION

Although the signaling system through heteromeric TGF-β receptors and Smad complexes is conceptually simple, combinatorial interactions provide a high degree of signaling specificity and versatility. The signaling responses can be qualitatively and quantitatively regulated by differential type I-type II receptor interactions, Smad complex formation, receptor and Smad interactions with accessory proteins, and crosstalk of the Smads with other signaling pathways. The specificity and quantitative regulation of Smad signaling has additional levels of versatility dictated by the complex nature of the Smad activator complex. In this complex, functional and physical interactions of Smads with DNA-specific transcription factors, which themselves are regulated by other signaling pathways, and transcription coactivators or corepressors that link the Smad complex to the Pol II complex, confer both specificity and complexity in transcriptional responses to TGF-β family ligands.

SUMMARY POINTS

1. As central signal transducers in TGF-β signaling, Smads transduce the signals from ligand-receptor complexes at the cell surface to gene transcription in the nucleus. Specific Smad-protein interactions determine signaling specificity.
2. The L45 loop in the type I receptor and L3 loop in R-Smads are the key determinants in specifying signaling in response to specific ligands.
3. Smads are weak DNA-binding proteins and naturally function by cooperating with a large number of sequence-specific DNA-binding transcription factors, thus leading to signaling versatility in TGF-β gene responses.
4. Inhibitory Smads, coreceptors at the surface, and intracellular kinases can modify the signaling strength of Smads.

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FUTURE ISSUES TO BE RESOLVED

1. A critical issue is how activated R-Smads are dephosphorylated, leading to recycling of Smads. What are the phosphatases?
2. It is important to solve the structures of full-length Smad proteins as well as those of Smad complexes with other transcriptional partners to understand how Smads function in transcriptional control.
3. Since Smads activate or repress transcription of genes in the context of chromatin, it is important to understand the effects of Smad signaling on chromatin remodeling.
4. Experimental approaches need to be improved to better understand the roles of endocytosis and intracellular routing in TGF-β signaling.
5. Are Smads the only signal transducers to receive signals directly from TGF-β receptors that lead to changes in transcription?
6. The mechanisms through which non-Smad signaling pathways are activated by the receptors and what these pathways contribute to the cellular response need to be better defined.

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