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Regulation of Smad Activity

Minireview

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Transforming growth factor-β (TGFβ) superfamily members are a group of secreted polypeptides that regulate a diverse array of developmental and biological processes. Disruption of the ligands or of components of their signaling pathways are associated with human diseases including cancer and hereditary conditions (Massagué, 1998). Superfamily members include TGFβs, activins/inhibins and the bone morphogenetic proteins (BMPs). These factors signal through heteromeric complexes of transmembrane type I and type II Ser/Thr kinase receptors. Within this complex the type II receptor kinase activates the type I receptor kinase, which subsequently propagates signals to the Smad pathway (Derynck et al., 1998; Massagué, 1998). Smad proteins play a critical role in transmitting TGFβ superfamily signals from the cell-surface to the nucleus (Derynck et al., 1998; Massagué, 1998). Smads have two conserved domains in their amino- and carboxy-terminal regions, termed the MH1 and MH2 domains, respectively, as well as a central proline-rich linker region. Smads are subdivided into three classes, the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads), and the inhibitory Smads (I-Smads) each of which have distinct functions. The R-Smads are phosphorylated by specific type I receptors on a carboxy-terminal SSXS motif. Thus, the TGFβ and activin type I receptors activate Smad2 and Smad3, whereas ALK1 and the BMP type I receptors ALK2, ALK3, and ALK6 target Smad1, -5, and -8. Specific R-Smad-receptor interactions are mediated by loop 3 in the MH2 domain of Smads and loop 45 in the type I receptor kinase (Massagué, 1998). A basic pocket that is present in the R-Smads may facilitate this interaction by providing a docking site for the phosphorylated GS domain of the activated type I receptor (Wu et al., 2000). Since this basic pocket is not found in Smad4, this may provide a possible structural explanation for the observation that Smad4 does not associate with activated type I receptors.

Once phosphorylated, R-Smads dissociate from the receptor, bind to Smad4, and enter the nucleus. In the nucleus, heteromeric complexes of Smads function as effectors of TGFβ signaling by regulating transcription from specific gene promoters. Smads can bind DNA directly but with low affinity and specificity and thus rely on interactions with other DNA-binding proteins to target specific genes for transcriptional regulation.

The TGFβ and Activin Pathways

The first Smad DNA-binding partner identified was FAST (forkhead activin signal transducer), a winged helix forkhead transcription factor, that regulates activin-dependent induction of the *Mix.2* gene in *Xenopus* and *goosecoid* in mouse. FAST binds constitutively to specific elements in the promoters of these genes, but cannot activate transcription. However, when activin or TGFβ signaling pathways are stimulated, a higher order complex containing FAST, Smad2, and Smad4 assembles on the DNA and transcription is strongly activated. Smad2 mediates interaction with FAST and recruits Smad4 into the nuclear complex. This allows the Smad4 MH1 domain to contact DNA at a Smad-binding element that lies adjacent to the FAST site and this stabilizes DNA binding by the ternary Smad–FAST complex. Smad3 can replace Smad2 in this complex and at the *Mix.2* element can activate transcription (Yeo et al., 1999), whereas at the *goosecoid* promoter Smad3 blocks activation (Labbé et al., 1998). This suggests that Smad-dependent activation of FAST target genes may be promoter dependent.

There are now many examples in which Smads cooperate with DNA binding partners to regulate transcription of TGFβ/activin target genes. For example, Smad2 and/or Smad3 can associate with c-Jun/c-Fos, ATF2, TFE3, PEBP2/CBF, and the vitamin D receptor (Figure 1). As for Smad/FAST-dependent activation of transcription, the R-Smad/Smad4 complex is recruited to specific promoters through the direct interaction of the R-Smad with these specific DNA binding partners (Derynck et al., 1998). Furthermore, regulation of these elements often requires direct binding of the Smad MH1 domain to adjacent DNA elements. Once recruited to specific elements, both R-Smads and Smad4 can activate transcription by recruiting the coactivators CBP/p300 and MSG1, respectively (Derynck et al., 1998). Alternatively, they can also recruit corepressors such as TGIF or Ski family members, which in turn bind histone deacetylases (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999; Wotton et al., 1999). Smads can also be blocked by interactions with the nuclear oncoproteins, Evi-1 or E1A (Derynck et al., 1998; Nishihara et al., 1999). Thus, Smads can both positively or negatively regulate transcription of specific genes in response to TGFβ family signaling.

The BMP Pathway

Much of the work on Smad nuclear function has concentrated on analysis of the TGFβ and activin signaling pathway. By comparison, little is known of how BMP signaling regulates transcriptional responses. However, one study has described a unique mechanism to regulate the *osteopontin* gene. Osteopontin is expressed in the osteoblast lineage and its expression is repressed by Hoxc-8, a homeodomain-containing transcription factor that binds to elements in the promoter (Shi et al., 1999). Smad1 binds to Hoxc-8 and prevents Hoxc-8 from binding to the promoter. As BMPs appear to block Hoxc-8-mediated transcriptional repression, this suggests that Smad1 may activate transcription by dislodging inhibitory Hoxc-8 from the promoter.

BMP signaling may also function through cooperative mechanisms similar to those characterized for the TGFβ-regulated Smads (Hata et al., 2000 [this issue of *Cell*]). The *Xenopus* homeobox gene *XVent2* is an immediate early target of BMP signaling during gastrulation.

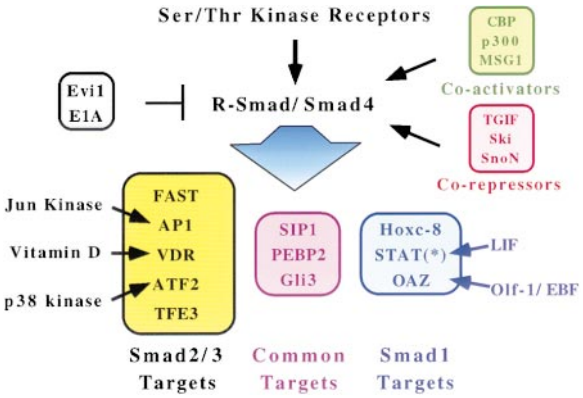

Figure 1. Nuclear Partners for Smads

In the nucleus, the R-Smad/Smad4 complex interacts with a variety of proteins to regulate transcription of target genes. Various DNA-binding proteins can interact specifically with either Smad1 or Smad2/3 whereas others, such as SIP1, PEBP2, and Gli3 are common targets of both R-Smads. In some cases, these DNA-binding proteins also receive inputs from other signaling pathways as indicated. Smads can then modulate gene expression by recruiting either coactivators or corepressors. Interactions with proteins such as Evi-1 and E1A can block Smad-mediated transcriptional activation. (*) Smad1 interaction with STAT is mediated by p300.

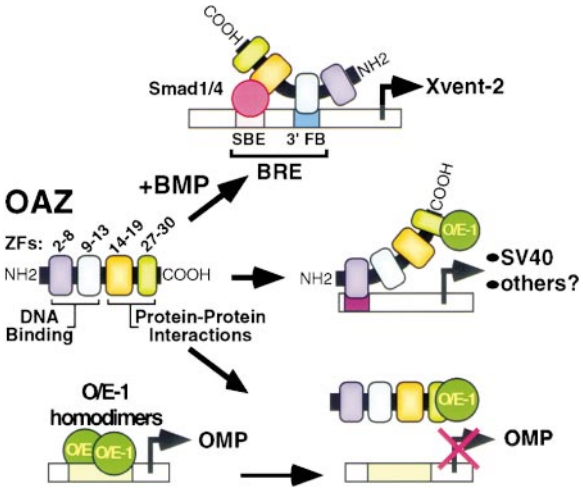

Figure 2. OAZ May Integrate Signals from Multiple Pathways

OAZ is a nuclear protein that contains 30 zinc finger domains. Two clusters of zinc fingers (ZF) mediate BMP responses by binding Smad1 and the 3′ flanking box in the XVent2 promoter (top). In addition, using different ZFs, OAZ can bind to the SV40 promoter and activate transcription in partnership with O/E-1 (middle). On their own, O/E-1 homodimers can activate target genes such as OMP and heterodimerization with OAZ blocks this activity.

Hata et al. identified a 52–base pair BMP response element (BRE) in the *Xvent2* promoter and showed that induction required both a Smad-binding element and a 3′ flanking box (3′ FB). Further, they found that the protein OAZ (for *O*lf-1/EBF *a*ssociated *z*inc finger) bound directly to the 3′ FB and was required for BMP-dependent induction of the BRE (Hata et al., 2000). OAZ has 30 Krüppel-type zinc fingers (ZF). Of these, ZF6–13 bind to the 3′ FB of the BRE and ZF14–19 bind to the Smad1 MH2 domain. In mammalian cells, efficient binding of OAZ to the BRE requires association with Smads and an intact SBE and 3′ FB. Hence, DNA binding by Smad likely cooperates with the weak affinity of full-length OAZ for the 3′ FB to promote binding of Smad-OAZ complexes to the BRE (Figure 2). Smads may then increase transcription by recruiting coactivators, such as CBP/p300.

OAZ can also bind a region in the SV40 minimal promoter using ZF2–8 (Tsai and Reed, 1997) and activates transcription by heterodimerizing through ZF27–30 with olfactory neuronal transcription factor, Olf-1 (Olf-1 is also called early B cell factor and will be referred to here as O/E-1). Interestingly, O/E-1 itself binds DNA as homodimers and regulates genes such as olfactory marker protein (OMP) that are expressed in terminally differentiated olfactory neurons. Furthermore, formation of OAZ-O/E-1 heterodimers can inhibit the formation of O/E-1 homodimers (Figure 2). Consequently, by inhibiting formation of O/E-1 homodimers, OAZ could negatively regulate differentiation of neuronal precursors in the olfactory epithelium. Together with the findings from Hata et al., these results show that OAZ can use different zinc finger domains to modulate transcriptional activity at distinct promoters. Thus, OAZ could function to integrate signals from multiple pathways to regulate patterning during development.

Generating Specific Smad Transcriptional Responses

The large number of Smad nuclear targets identified thus far begins to raise questions about how specific transcriptional programs are induced by TGFβ/activin or BMP signaling in a temporally and spacially restricted manner. Restricted expression of Smad nuclear targets can allow certain cell-type-specific responses. For instance, FAST and OAZ display limited expression patterns, and in cells not expressing these Smad partners target genes remain silent in response to TGFβ family signaling.

Other Smad partners are relatively ubiquitous, regulate transcription of target genes independent of Smad activity and are controlled by other signaling pathways. Input from these other pathways often is required before Smads can regulate the activity of these factors. For example, activation of AP1-containing elements by Smads requires activation of c-Jun by Jun kinase, which is activated by a large number of external cues that may include TGFβ in some systems (Hocevar et al., 1999). Similar cooperativity has been described for ATF2 and the p38 kinase cascade and for the vitamin D receptor. In these examples, Smad input represents a secondary signal that modifies the output from the primary signaling pathway. Further, efficient activation of specific promoters often requires that Smad binding sites lie adjacent to the site bound by these Smad partners. Consequently, both promoter and cellular context can play an important role in defining the transcriptional program manifested by Smad signaling pathways.

The amount of Smad protein that enters the nucleus is another important determinant of specificity in the transcriptional response. Considerable progress in understanding how the magnitude of TGFβ signaling affects the biological outcome has been made in *Xenopus*. For instance, variations in either activin or Smad2 levels

alter cell fate decisions, and this correlates with differential transcriptional responses. Thus, the concentration of nuclear Smad is an important determinant of the transcriptional outcome. It is unknown how this occurs, but one possibility is that DNA binding partners compete for Smads. When Smads are abundant, this may not be significant and a wide range of transcriptional responses to ligand may be manifested. However, when Smad levels are limiting, competition among Smad binding partners may result in activation of only a subset of target genes. This would provide a mechanism whereby Smads can translate different ligand concentrations into specific transcriptional responses.

Regulation of Smad Levels and Subcellular Distribution

Smad signaling should not be considered as simply being on or off, but rather should be thought of quantitatively, in terms of how much signaling is occurring. Consequently, controlling the level of nuclear Smad protein can have an important influence on the biological activity of the pathway. Several pathways appear to control nuclear Smad levels either by regulating protein turnover, both prior to and after entry into the nucleus, or by regulating how Smads access the receptor kinase.

Smad Turnover. The ubiquitin–proteasome pathway functions to control a wide range of cellular functions by selectively targeting proteins for degradation. Ubiquitination of proteins is mediated by E3 ligases, which recruit target proteins to the ubiquitin conjugation machinery. An E3 ubiquitin ligase called Smurf1 that preferentially binds to the BMP-regulated Smads, Smad1, and Smad5 has been identified (Zhu et al., 1999). Smurf1 induces the ubiquitination and proteasome-mediated degradation of these Smads and antagonizes BMP signaling in *Xenopus*. Smurf1 contains a HECT domain, a C2 domain, and two WW domains. WW domains mediate protein–protein interactions by binding to PY motifs in target proteins. A PY motif is present in the linker region of R-Smads and I-Smads and in Smad1 it mediates interactions with Smurf1. Smurf1 recognizes Smad2 and Smad3 only poorly and in *Xenopus* does not antagonize, but rather enhances Smad2-dependent signals. Proteolysis of the BMP-regulated Smads thus appears to increase responsiveness to Smad2. Hence, Smurf1-mediated pathways can control the competence of cells to respond to both BMP and TGFβ/activin by directly regulating the level of Smad1 protein in the cells.

To reduce TGFβ or BMP signaling, Smad complexes must be removed from the nucleus. Interestingly, activation of Smad2 by TGFβ signaling induces its ubiquitin-mediated proteolysis (Lo and Massagué, 1999). This degradation is not dependent on phosphorylation of Smad2, *per se*, but occurs when Smad2 enters the nucleus. Thus, nuclear Smads can be downregulated by ubiquitin-mediated proteolysis. Ubiquitin-mediated proteolysis of Smad partners may also play a role in TGFβ signaling. Two nuclear oncoproteins, Ski and SnoN, are partners for Smad2 and Smad3 and function as corepressors to antagonize TGFβ signaling. Activation of TGFβ signaling appears to induce the rapid degradation of SnoN by the ubiquitin–proteasome system (Stroschein et al., 1999; Sun et al., 1999). Although the mechanism remains unknown, degradation of SnoN may be an important step during the initiation of Smad-dependent TGFβ signaling.

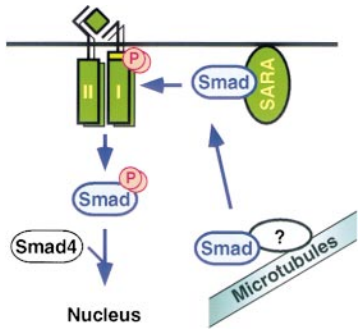

Figure 3. Regulation of Smad Localization

Smad subcellular localization is determined by interactions of unphosphorylated Smads with microtubule networks and through association with SARA. This may represent a pathway that controls trafficking of Smad2 and Smad3 to the TGFβ receptor.

Regulation of Smad Access to Receptors. Regulation of the subcellular localization of Smads appears to be a critical aspect of this signaling pathway, and work on anchoring proteins that bind unphosphorylated Smads is beginning to shed light on a pathway that may control Smad access to the transmembrane receptor kinase. SARA (for *S*mad *a*nchor for *r*eceptor *a*ctivation) binds unphosphorylated Smad2 and Smad3 and contains a FYVE domain, which can bind phosphatidylinositol-3-phosphate (Tsukazaki et al., 1998). In SARA, the FYVE domain is required for its proper subcellular localization in punctate regions that also contain the TGFβ receptors. SARA recruits Smad2 into these regions and may facilitate signaling by bringing the Smad substrate to the receptor. Activation of TGFβ signaling causes phosphorylated Smad2/3 to dissociate from SARA and permits Smad nuclear accumulation. The SARA–Smad interaction is mediated by a proline-rich coil, an α helix and a β strand in the SARA SBD that bind cooperatively with an extended interaction surface on the Smad2 MH2 domain (Wu et al., 2000). Furthermore, mutation of an asparagine residue in Smad2 that contacts the SBD interferes with Smad2 binding to SARA and appears to inhibit Smad2-dependent signaling. This, together with the observation that mutants of SARA that mislocalize Smad2 also inhibit TGFβ signaling, suggests that regulating Smad2 localization prior to activation of the pathway is an important feature of TGFβ signaling.

Smads and Microtubules. Smad2/3 and Smad4 may also be distributed along the microtubule (MT) network (Dong et al., 2000). Smad2 associates with β-tubulin, a major component of MTs and activation by TGFβ results in dissociation from β-tubulin and the MT network. This occurs concomitantly with Smad2 phosphorylation and nuclear translocation and suggests that phosphorylation of Smads may prevent interaction with the MT network. It is not known whether Smad2 binds directly to β-tubulin, however, disrupting MTs using the drug nocodazole also interferes with Smad2 association with β-tubulin, so binding to MTs may occur through accessory molecules. Interestingly, brief treatment of cells

with nocodazole resulted in activation of Smad-dependent transcriptional responses and caused phosphorylation of Smad2 on the carboxy-terminal SSXS motif in the absence of added TGFβ. These effects are probably due to destabilization of the MT network, because taxol, which counteracts nocodazole to stablize MTs, prevents nocadazole-dependent activation of Smads. Furthermore, colchicine and a mutant of α-tubulin, which both interfere with MT networks, also activated Smad signaling. These results suggest that microtubules sequester Smads from the receptor prior to TGFβ stimulation of cells.

One question that arises from this work is how Smads get from MTs to the receptor. There is no obvious correlation between the location of the MT network and the punctate subcellular distribution displayed by the TGFβ receptors. However, one possibility is that SARA may facilitate movement of Smads to the receptor by recruiting Smad2 from the MT network (Figure 3). These studies are thus beginning to shed light on a possible pathway that controls access of R-Smads to the receptors prior to their phosphorylation and nuclear translocation.

An intriguing observation that has emerged from investigation of MTs is that brief nocodazole treatment induces phosphorylation of Smad2 at the COOH-terminal SSXS motif in the absence of TGFβ and enhances TGFβ-dependent phosphorylation of Smad2. It is unclear whether nocodazole-dependent phosphorylation of Smad2 occurs through TGFβ receptor complexes. However, these results suggest that the MT network imposes constitutive negative regulation on Smad activation in unstimulated cells. How TGFβ binding to the receptor complex overrides this inhibition to activate R-Smads is unknown. Nevertheless, negative regulation of Smad signaling adds an important dimension to the growing list of cellular processes that are controlled by the MT network. This could have important implications for diseases in which MT stability is affected as some of the pathology might arise through promiscuous activation of the Smad signaling pathway.

Concluding Remarks

The Smads have emerged as a family of transcriptional comodulators that interact with a wide range of DNA-binding proteins to regulate their activity in response to TGFβ family signaling. Smad function in this regard is intimately linked to how much Smad is in the nucleus and this is subjected to extensive regulation both prior to and after activation by the receptor kinase. Thus, the study of Smad regulation has taken an interesting turn toward understanding how Smad proteins are turned over and how they are trafficked about the cell.

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