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Signal Transduction by Transforming Growth Factor-β: A Cooperative Paradigm With Extensive Negative Regulation

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Abstract Transforming growth factor-β (TGF-β) represents an evolutionarily conserved family of secreted factors that mobilize a complex signaling network to control cell fate by regulating proliferation, differentiation, motility, adhesion, and apoptosis. TGF-β promotes the assembly of a cell surface receptor complex composed of type I (TβRI) and type II (TβRII) receptor serine/threonine kinases. In response to TGF-β binding, TβRII recruits and activates TβRI through phosphorylation of the regulatory GS-domain. Activated TβRI then initiates cytoplasmic signaling pathways to produce cellular responses. SMAD proteins together constitute a unique signaling pathway with key roles in signal transduction by TGF-β and related factors. Pathway-restricted SMADs are phosphorylated and activated by type I receptors in response to stimulation by ligand. Once activated, pathway-restricted SMADs oligomerize with the common-mediator Smad4 and subsequently translocate to the nucleus. Genetic analysis in *Drosophila melanogaster* and *Caenorhabditis elegans*, as well as TβRII and SMAD mutations in human tumors, emphasizes their importance in TGF-β signaling. Mounting evidence indicates that SMADs cooperate with ubiquitous cytoplasmic signaling cascades and nuclear factors to produce the full spectrum of TGF-β responses. Operating independently, these ubiquitous elements may influence the nature of cellular responses to TGF-β. Additionally, a variety of regulatory schemes contribute temporal and/or spatial restriction to TGF-β responses. This report reviews our current understanding of TGF-β signal transduction and considers the importance of a cooperative signaling paradigm to TGF-β-mediated biological responses. J. Cell. Biochem. Suppls. 30/31:111–122, 1998.

Key words: TGF-β cooperative signaling; SMADs

Transforming growth factor-β (TGF-β) is the prototype for an extensive family of growth and differentiation factors that also includes the activin/inhibins and bone morphogenetic proteins (BMPs) [Heldin et al., 1997]. While initial interest in TGF-β arose out of its stimulation of anchorage-independent growth in mesenchymal cells [Tucker et al., 1984] and its potent growth inhibitory effects on epithelial cells [Heldin et al., 1997], we now recognize its influential role in cell fate determination and tissue morphogenesis. This occurs in part through its control of cell proliferation, differentiation, motility, adhesion, and apoptosis. Given its wide range of effects, tremendous effort has been expended to uncover the mechanisms by which TGF-β communicates its instructions to the nucleus. Recently, there has been significant advances in our understanding of these mechanisms (Fig. 1). These include the cloning and characterization of transmembrane receptors for TGF-β family members and numerous receptor interacting proteins. Equally important has been the identification of ligand specific, cytoplasmic effectors, the SMADs, that in cooperation with ubiquitous transcriptional activators and the signaling pathways that regulate them, control expression of target genes. Collectively, these factors constitute a communication network exploited by TGF-β family members to regulate gene expression, and suggest a paradigm in which signaling pathways activated by ligand binding and operating in parallel, converge at target promoters to produce ligand specific responses. This review introduces essen-

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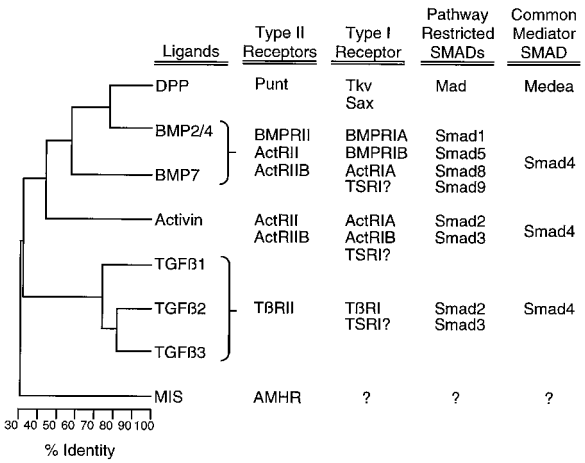

Fig. 1. The TGF-β superfamily, receptors, and SMAD proteins. The evolutionary relationship for representative ligands of the TGF-β superfamily is shown, as well as the corresponding signaling receptors and SMAD proteins. All are mammalian factors except for Dpp and its downstream signaling components. TSR1 can bind members of TGF-β, activin, and BMP families of ligands, but its function in signaling remains unknown.

tial components of this network and describes their interplay in a cooperative signaling paradigm.

TGF-β SIGNALING RECEPTORS: A SENSOR/TRANSDUCER COMPLEX

TGF-β family members transduce signals through a conserved group of transmembrane serine/threonine (Ser/Thr) kinase receptors [Heldin et al., 1997]. Sequence comparisons indicate that these receptors fall into two subgroups, designated types I and II, which parallels their distinct roles in signal transduction. Each receptor is characterized by a cysteine-rich ectodomain that interacts with ligand, a single transmembrane spanning α-helix, and a cytoplasmic region dominated by the kinase domain. Unique among the type I receptors is the GS-domain, a stretch of alternating glycine and Ser/Thr residues within the juxtamembrane region, immediately N-terminal to the kinase domain. The type II receptor is a constitutively active kinase, otherwise unaffected by ligand binding. In contrast, the kinase activity of the type I receptor is ligand inducible.

Significant insights into receptor activation by TGF-β have come from studies of mink lung epithelial cells lacking either TGF-β type I (TβRI) or type II (TβRII) receptors and whose TGF-β responsiveness could be restored by receptor replacement [Wrana et al., 1994]. These experiments indicate that cellular responses to TGF-β require distinct but complementary contributions from TβRI and TβRII (Fig. 2). TβRII fulfills the role of "sensor": while alone capable of binding ligand, it cannot elicit responses to TGF-β independently. Conversely, TβRI acts as a "transducer": while necessary for ligand-mediated responses, it does not alone bind TGF-β. Instead, ligand binding to TβRII recruits TβRI into a heteromeric complex. Interactions between the cytoplasmic domains of the two receptor types further stabilize this complex [Feng and Derynck, 1996]. The juxtaposition of TβRII and TβRI permits transphosphorylation of TβRI within its GS-domain. TβRI phosphorylation activates its kinase activity toward downstream effectors [Wrana et al., 1994]. Mutational analysis of TβRI has reinforced this model [Weiser et al., 1995]. Eliminating phospho-acceptor sites within the GS-domain inhibits transcriptional and growth inhibitory responses to TGF-β. Furthermore, replacement of Thr-204 with aspartate creates a constitutively active TβRI kinase that elicits TGF-β responses in the absence of either TGF-β or TβRII. A highly divergent nine amino acid region among type I receptors, the L45 loop, has been shown to determine type I receptor signaling specificity [Feng and Derynck, 1997].

Evidence indicates that the functional receptor is a heterotetramer composed of two TβRI and two TβRII molecules. This was demonstrated at a biochemical level using ^{125}I-TGF-β receptor cross-linking, followed by immunoprecipitation and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Yamashita et al, 1994]. The functional significance of the heterotetrameric structure is suggested by complementation of activation-deficient and kinase-deficient TβRI mutants in signaling [Weis-Garcia and Massagué, 1996]. Moreover, using chimeric receptors composed of the extracellular domain of the erythropoietin receptor and the cytoplasmic domains of TβRI and TβRII, it was shown that both homomeric and heteromeric receptor interactions are required for TGF-β-mediated growth inhibition [Luo and Lodish, 1996].

The segregation of TβRI and TβRII in the absence of TGF-β is functionally significant. Unlike traditional receptor tyrosine kinase signaling, where both receptor dimerization and activation are mediated by ligand binding, the ligand binding component of the TGF-β recep-

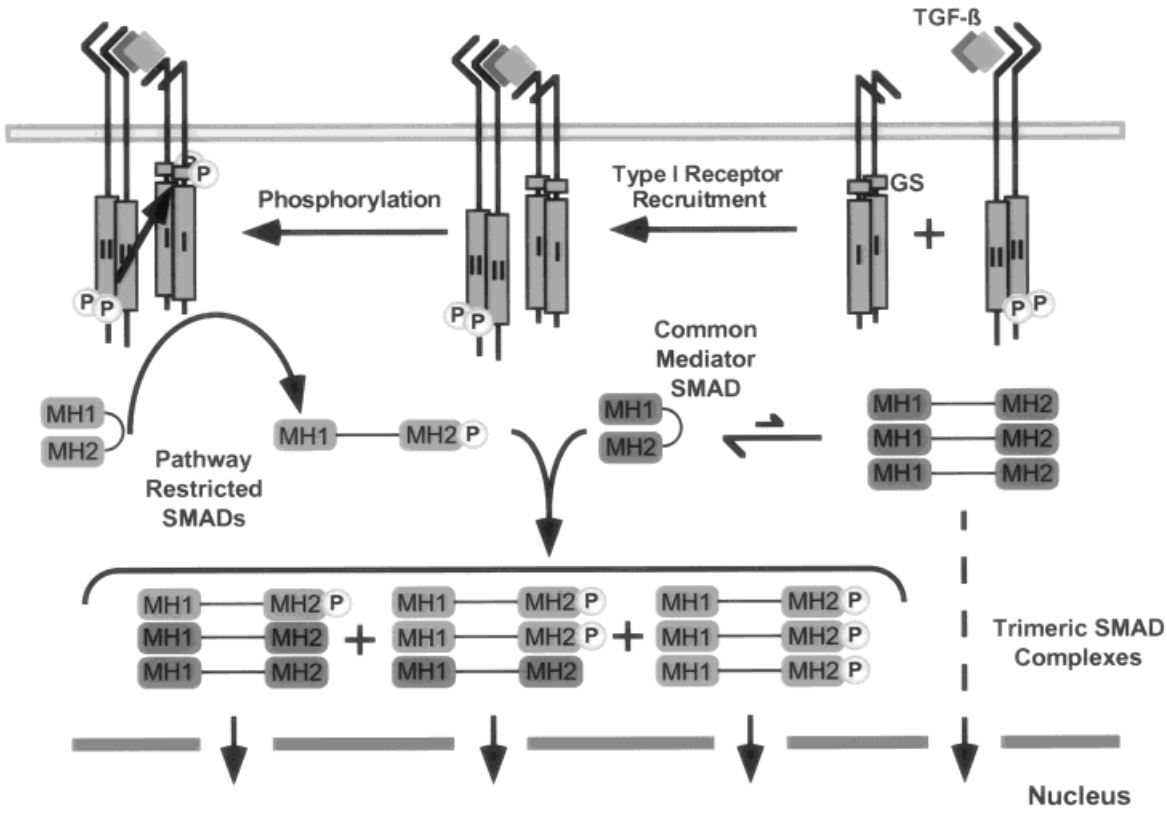

Fig. 2. TGF-β activates a heteromeric receptor complex which initiates oligomerization of SMADs. TGF-β binds independently to TβRII. Through its constitutively active kinase domain, TβRII transphosphorylates TβRI in its regulatory GS-domain. Monomeric pathway-restricted Smads are phosphorylated at their C-terminus by activated TβRI. This modification relieves reciprocal, MH1–MH2 domain inhibition and promotes the formation hetero-oligomers, most likely heterotrimers, with the common-mediator Smad4. Variations in oligomer stoichiometry may exist, as well as potentially higher-order oligomers. The heterotrimeric SMAD complexes translocate to the nucleus. **Color plate on page 319.**

tor complex, TβRII, is constitutively dimerized and its kinase domain constitutively active. Maintaining functional separation between TβRII and TβRI is essential to prevent ligand independent activation of TGF-β signaling. Thus, TGF-β can be thought of as an adapter that promotes assembly and stabilization of the "sensor"-"transducer" complex. While variations on this theme have been described, evidence suggests the likelihood that other TGF-β superfamily members activate their receptors in a similar manner.

RECEPTOR INTERACTING PROTEINS

Proximal signaling events coupling TGF-β receptor activation to biological responses are likely to involve proteins that directly bind the receptor complex. Several candidate receptor binding proteins have been identified by interaction cloning using a yeast two-hybrid strategy. FKBP12, a binding protein for the macrolide immunosuppressant FK506, interacts with a Leu-Pro motif in the GS-domain of TβRI and other type I receptors [Wang et al., 1996; Chen et al., 1997]. This interaction is disrupted by ligand binding. While dispensable for TGF-β signaling and not a direct substrate for TβRI kinase activity, blocking the interaction between FKBP12 and TβRI potentiates TGF-β responses. The same effect was seen in signaling by müllerian inhibiting substance, suggesting that FKBP12 inhibits signaling generally by TGF-β family members [Wang et al., 1996]. Because FKBP12 interacts with ligand-free TβRI and is liberated by ligand binding, it is thought to prevent spontaneous type I receptor activation, helping to insure the functional separation described above.

In addition to FKBP12, other type I receptor interacting proteins include the α-subunit of type I isoprenyltransferases (FTα) [Kawabata et al., 1995], *Drosophila* inhibitor of apoptosis (DIAP)-1 and -2 [Oeda et al., 1998], and TβRI-associated protein (TRAP)-1 and -2 [Charng et

al., 1998]. The WD-domain protein TRIP-1 was also identified through its interaction with TβRII [Chen et al., 1995]. Interestingly, TRAP-1 could discriminate between quiescent TβRI and TβRI activated either by mutation or by ligand binding [Charng et al., 1998]. Thus, TRAP-1 may act as an adapter protein that helps to recruit signal transducers and regulators to the activated receptor complex. The functional significance of each of these factors in TGF-β signaling remains to be determined.

SMAD PROTEINS COUPLE RECEPTOR ACTIVATION TO THE NUCLEUS

Efforts to identify components of the signaling machinery downstream of Ser/Thr kinase receptors have been propelled by studies in genetically tractable organisms. These studies led to the discovery of SMAD proteins. The founding member of the SMAD family, *Mothers against dpp* (*Mad*) was identified as a dominant enhancer of weakly mutant alleles of *decapentaplegic*, a BMP homologue in *Drosophila melanogaster* [Raftery et al., 1995; Sekelsky et al., 1995]. Genetic screens in *Caenorhabditis elegans* for mutant phenotypes like those observed for Ser/Thr kinase receptors *daf-1* and *daf-4* revealed three genes, *sma-2*, *sma-3*, and *sma-4*, with homology to *Mad* [Savage et al., 1996]. The evolutionary conservation of these proteins, and their position downstream of both a TGF-β family member and Ser/Thr kinase receptors prompted a search for homologous factors in vertebrates.

To date, nine vertebrate SMADs have been identified [Attisano and Wrana, 1998] (Fig. 1). They are characterized by homologous regions at their N- and C-termini known as Mad homology (MH)-1 and MH2 domains, respectively. A divergent linker region separates the these domains. Both structural and functional differences provide the basis for a division of the SMADs into three groups: pathway-restricted, common-mediator, and inhibitory SMADs. Upon receptor activation, SMADs oligomerize in the cytoplasm and subsequently translocate to the nucleus to participate in gene expression (Fig. 2). These oligomers are composed of pathway-restricted SMADs and the common-mediator Smad4. However, in the absence of Smad4, the pathway-restricted Smads 2 and 3 can form both homo- and hetero-oligomers with each other [Kawabata et al., 1998]. While evidence indicates that hetero-oligomeric complexes between pathway-restricted SMADs and Smad4 mediate ligand specific responses, the function of Smad4 deficient oligomers remains to be determined.

Pathway-Restricted SMADs

As their name implies, the pathway-restricted SMADs are activated in response to particular TGF-β family members (Fig. 1). Smad1, Smad2, Smad3, Smad5, Smad8, and Smad9/MADH6 constitute the pathway-restricted SMADs [Attisano and Wrana, 1998]. These proteins interact with, and are substrates for, activated type I receptors. Smad2 and Smad3 are phosphorylated after both TGF-β and activin stimulation [Zhang et al., 1996; Nakao et al., 1997a], while Smad1, and presumably Smad5, Smad8, and Smad9/MADH6 are similarly modified through BMP exposure [Hoodless et al., 1996; Kretzschmar et al., 1997a]. Several observations support the notion that pathway-restricted SMADs are direct substrates for activated type I receptor kinases and that this phosphorylation is required for ligand specific functions. With in vitro assays, Smad1 and either Smad2 or Smad3 are phosphorylated by purified BMPRI [Kretzschmar et al., 1997a] and TβRI [Macias-Silva et al., 1996; Zhang et al., 1996], respectively. Phosphopeptide mapping of in vitro phosphorylated Smad1 displays the same pattern as that observed in vivo after agonist stimulation. In each case, SMAD phosphorylation occurs in an -SSXS motif conserved in the C-termini of pathway-restricted SMADs. Additional evidence for direct phosphorylation of pathway-restricted SMADs by type I receptors came from coprecipitation experiments. Using ^{125}I-TGF-β cross-linking, Smad2 was shown to coprecipitate ligand-bound receptors only in cells coexpressing wild-type TβRII and kinase-deficient TβRI [Macias-Silva et al., 1996]. No interaction was observed either when both receptors were wild-type or when the TβRII kinase was inactivated. These results indicate that Smad2 transiently associates with TβRI that has been activated by TβRII. This conclusion is supported by the observation that phosphorylation incompetent mutants of Smad2 form stable complexes with wild-type TGF-β receptors, fail to translocate to the nucleus following TGF-β exposure, and antagonize TGF-β-induced transcription.

That pathway-restricted SMADs are used by particular ligands is supported by their different effects in *Xenopus* microinjection assays.

Xenopus Smad1 (Xmad1), human Smad1 and *Drosophila* Mad induce ventral mesoderm formation, a characteristic response to BMP [Graft et al., 1996; Thomsen, 1996]. By contrast, Smad2 from *Xenopus*, mouse, and human will elicit dorsal mesoderm, mimicking activin, Vg-1, or TGF-β [Graft et al., 1996]. Collectively, these results support the hypothesis that in response to a specific ligand, a unique SMAD is recruited to the receptor complex and activated by phosphorylation of its -SSXS motif by the type I receptor. The activated SMADs enter the nucleus as part of an oligomeric complex to influence transcription of target genes.

Common Mediator SMADs

Of the SMADs so far identified, only Smad4 and its homologues in *C. elegans* and *D. melanogaster* come under this heading. Smad4 has no C-terminal -SSXS motif and is not a substrate for type I receptors [Zhang et al., 1996; Nakao et al., 1997a; Lagna et al., 1996]. Instead, Smad4 acts as a signaling partner for pathway-restricted SMADs activated by agonist stimulation [Zhang et al., 1996; Nakao et al., 1997a; Lagna et al., 1996; Kretzschmar et al., 1997]. Once activated, pathway-restricted SMADs form hetero-oligomers with Smad4. This complex translocates to the nucleus to regulate gene transcription. Originally described as a putative tumor suppressor gene frequently mutated in pancreatic cancers (DPC4) [Hahn et al., 1996], Smad4 has since been implicated in TGF-β, activin, and BMP signaling through its interaction with Smad2/Smad3, and Smad1, respectively [Zhang et al., 1996; Nakao et al., 1997a; Lagna et al., 1996]. Its ability to cooperate with a broad range of pathway-restricted SMADs underscores its critical role in signal transduction by TGF-β and related factors.

Inhibitory SMADs

TGF-β family members elicit pleiotropic responses that impact cell fate. Given the central role of SMADs in signaling by these factors, it is reasonable to expect that their functions might be negatively regulated. One mode of negative regulation is exhibited by the inhibitory SMADs, Smad6 and Smad7 [Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997b], and the related *Daughters against decapentaplegic* (*Dad*) [Tsuneizumi et al., 1997] from *D. melanogaster*. While sharing homology with other SMADs in their C-termini, the inhibitory SMADs diverge significantly in their N-terminal domains. Also of note, the inhibitory SMADs lack a C-terminal -SSXS motif. Smad7 inhibits TGF-β signaling in cultured cells. It stably interacts with activated TβRI, preventing Smad2/3 phosphorylation, blocking both association with Smad4 and Smad2/3 nuclear accumulation. Smad6 also associates with activated type I receptors for TGF-β, activin, and BMP and inhibits phosphorylation of Smad2 and Smad1. Whether competition with pathway-restricted SMADs for type I receptors reflects the primary mechanism of inhibitory SMAD function is unclear. Alternate mechanisms, such as formation of complexes with other SMADs [Topper et al., 1997], have been described. Furthermore, the degree of functional overlap among the inhibitory SMADs has not been explored.

Interestingly, the expression of Smad7 can be regulated at the transcriptional level by TGF-β [Nakao et al., 1997b], suggesting the existence of a feedback loop, that in concert with other regulators (see below) imposes temporal and/or spatial restrictions on TGF-β signaling through the SMAD pathway. Similarly, *Dad* expression is potentiated by Dpp in *D. melanogaster* [Tsuneizumi et al., 1997]. These and other mechanisms of negative regulation are promising avenues for new exploration.

Oligomerization of SMADs

The Smad4 MH2 domain expressed in bacteria adopts a homotrimeric structure in solution [Shi et al., 1997], and by extension, other SMADs have been presumed to adopt a similar structure. Furthermore, the ability of pathway-restricted SMADs to form hetero-oligomers with Smad4 in response to receptor activation prompted a heterohexameric model for activated SMAD complexes. However, recent data suggest that in vivo, SMADs are predominantly monomeric, but form homo- and heterotrimeric complexes in response to receptor activation [Kawabata et al., 1998] (Fig. 2). Homotrimers of Smad2 and Smad3, and heterotrimeric complexes between them, form independent of Smad4. Yet when Smad4 was present, Smad2 and Smad3 formed primarily heterotrimeric, Smad4-containing complexes. Moreover, Smad4 competed with Smad2 for inclusion in these complexes. These observations suggest that cells preferentially assemble heteromeric complexes between Smad4 and pathway-restricted SMADs

in response to ligand, and that assembly of oligomeric complexes of potentially variable composition may be an important event in signaling by TGF-β- related proteins. The functional significance of homotrimeric SMADs in vivo remains to be determined, but may constitute a mechanism for controlling SMAD bioavailability following specific stimuli.

SMAD Domain Functions and Regulation by Intrinsic and Extrinsic Mechanisms

Dissection of SMAD protein structure has provided insights into the functions of individual SMAD domains. By virtue of its ability to impart transactivating activity to fusion proteins with the Gal4 DNA-binding domain, the MH2 region of pathway-restricted SMADs is considered an effector domain [Liu et al., 1996]. This characteristic may reflect extensive involvement of the MH2 domain in protein–protein interactions, particularly with other transactivating factors. For example, interaction between Smad2 and the winged-helix transcription factor FAST-1 occurs within the MH2 domain [Chen et al., 1996], and indeed the Smad2 MH2 domain alone can mimic induction of dorsal mesoderm by activin in *Xenopus* embryos [Baker and Harland, 1996]. Recently, an interaction between the transcriptional coactivator CBP/p300 (see below) and the MH2 domain of receptor activated Smad3 has been described [Feng et al., 1998; Janknecht et al., 1998]. Additionally, the MH2 domains are responsible for homomeric and heteromeric interactions between SMADs [Zhang et al., 1997].

Without a specific stimulus, SMAD proteins reside in the cytoplasm. When stimulated, they translocate to the nucleus as part of an oligomeric complex [Attisano and Wrana, 1998]. The observation that MH1 domain deletion from Smad2 results in constitutive nuclear localization [Baker and Harland, 1996] suggests an intrinsic inhibitory role for the MH1 domain in signaling by pathway-restricted SMADs. This conclusion is supported by the observation that MH1 and MH2 domains interact directly in the basal state, preventing the formation of active oligomers [Hata et al., 1997]. Moreover, an MH1 domain antagonizes biological responses mediated by the corresponding MH2 domain. Intrinsic inhibition of SMAD function mediated by the MH1 domain is relieved by agonist induced phosphorylation of the -SSXS motif, which presumably antagonizes the intramolecular MH1–MH2 interaction. The significance of this intrinsic negative regulation is underscored by naturally occurring mutations in the MH1 domains of both Smad2 and Smad4 that enhance affinity for the MH2 domain, thus attenuating SMAD-mediated growth inhibition [Eppert et al., 1996; Schutte et al., 1996].

The MH1 domain has also been implicated in direct DNA binding, which may be essential for some transcriptional responses. The MH1 domain of Mad is necessary and sufficient for binding to the "quadrant" enhancer of the *vestigial* (*vg*) wing patterning gene in *D. melanogaster* [Kim et al., 1997]. Similarly, Mad binds to the Dpp response element in the *Ultrabithorax* (*ubx*) promoter via its MH1 domain. Recently, Dennler et al. [1998] described the direct interaction of Smad3 and Smad4 with a CAGA-box, a DNA element repeated three times in the TGF-β responsive regions of the plasminogen activator inhibitor (PAI)-1 promoter. This interaction requires the MH1 domain of both SMADs. Smad3 additionally requires either agonist stimulation or MH2 domain deletion. An inverted repeat CAGA-box motif with TGF-β-inducible Smad3/Smad4 binding has also been described in the *JunB* promoter [Jonk et al., 1998]. Again, Smad3 binding required either deletion of the MH2 domain or TGF-β stimulation. These data imply that an activation induced conformational change is needed for Smad3 DNA binding, and more generally suggests intrinsic inhibition of MH1 domain DNA binding by the MH2 domain. Together, these data argue that in a basal state, MH1 and MH2 domains provide intrinsic, reciprocal inhibition that is liberated by receptor activation and -SSXS phosphorylation.

In addition to intrinsic regulatory mechanisms, recent reports also describe extrinsic factors that contribute to SMAD regulation. The ERK MAP kinase pathway is activated in response to mitogenic growth factors such as epidermal growth factor (EGF) that signal through receptor tyrosine kinases [Denhardt, 1996]. ERK-mediated phosphorylation of target transcriptional regulators contributes to the mitogenic influence of these factors. Recently, multiple serine residues in the linker region of Smad1 were shown to be phosphorylated by ERK, both in vitro and in vivo in response to EGF [Kretzschmar et al., 1997b]. While phosphorylation of Smad1 by ERK was independent of -SSXS phosphorylation and did not affect

association with Smad4, it did antagonize nuclear translocation of the SMAD oligomeric complex in response to BMP stimulation. Insofar as biological responses reflect an altered balance of signaling through multiple pathways, ERK mitogenesis may involve simultaneous potentiation of growth promoting pathways and attenuation of growth inhibitory pathways. Moreover, the presence of additional consensus motifs for MAP kinases in the linker regions of SMAD proteins suggests that more extensive extrinsic regulation of SMAD signaling may occur in this manner.

In addition to regulatory phosphorylation of SMADs, a Ca^{2+} dependent interaction between calmodulin (CaM) and several SMAD family members has been described [Zimmerman et al., 1998]. CaM bound the N-terminal half of Xmad2 between residues 76 and 208. Both CaM coexpression and a CaM-specific antagonist suggested a negative regulatory role for CaM in both activin and TGF-β signaling in a transient assay. Given the wide array of factors regulated by CaM either directly via protein–protein interaction or indirectly by CaM-dependent kinases, it is attractive to speculate that CaM influences SMAD protein function in response to agents that regulate intracellular Ca^{2+} flux.

Negative regulation of Smad3 by a distinct mechanism was recently described for Evi-1, a zinc-finger domain protein implicated in leukemic transformation [Kurokawa et al., 1998]. Evi-1 interacts directly with Smad3; this interaction is enhanced by TGF-β exposure. Enhanced binding is consistent with the nuclear localization of Evi-1 and suggests that Evi-1 binds Smad3-containing oligomers. Evi-1 potently inhibits TGF-β-mediated transcriptional and growth inhibitory responses. Targeting of nuclear SMAD complexes by Evi-1 is distinct from regulation described above, which affect events preceding nuclear entry. Together with inhibitory SMADs, these modes of regulation help to restrict SMAD function both before and after activation.

TRANSCRIPTIONAL REGULATION BY TGF-β: A COOPERATIVE SIGNALING PARADIGM

Before the discovery of SMAD proteins as essential for signaling by TGF-β family members, the TGF-β-responsive regions within promoters of several genes had been defined and suggested the involvement of other ubiquitous transcriptional regulators in TGF-β-mediated gene expression. For example, TGF-β induction of the PAI-1 and human collagenase promoters involves the transcription factor AP1 [Keeton et al., 1991; de Groot and Kruijer, 1990], whereas TGF-β-mediated transactivation of cyclin-dependent kinase inhibitors p21 [Datto et al., 1995] and $p15^{INK4B}$ [Li et al., 1995] involves SP1 function. However, it is unclear how activation of the TGF-β receptor is communicated to these nuclear factors. Significant insights have come from studies of the activin-inducible *Mix.2* gene from *X. laevis* [Chen et al., 1996]. A hexanucleotide repeat motif within the activin response element (ARE) of the *Mix.2* promoter binds FAST-1, a member of the forkhead/winged-helix family of transcription factors required for activin-dependent *Mix.2* induction. FAST-1 is constitutively bound to the ARE. In response to activin stimulation, FAST-1 becomes part of a larger ARE-bound complex known as the activin response factor (ARF). In addition to FAST-1, ARF also contains Smad2 and Smad4. In assembling ARF, Smad2 interacts directly with FAST-1 and is required for Smad4 recruitment. FAST-1 is thought to contribute the primary DNA binding activity for ARF. However, Smad4 is thought to stabilize DNA binding through its MH1 domain and to activate transcription via its MH2 domain. Thus, functions contributed by SMADs and FAST-1 are necessary for activin- inducible expression of *Mix.2*.

Activin induction of the *Mix.2* gene provides a conceptual framework to reconcile contributions from both SMADs and ubiquitous transactivators in gene expression mediated by TGF-β and related factors (Fig. 3). The TGF-β-responsive regions of the human PAI-1 promoter contain DNA elements which show strong homology to the consensus binding site for the ubiquitous transcription factor AP1 [Keeton et al., 1991]. Inactive components of the JNK pathway, which regulates AP1 activity, inhibit TGF-β induction of 3TP-Lux, a reporter that contains elements of the PAI-1 promoter [Atfi et al., 1997]. Moreover, Rho proteins, known agonists of the JNK pathway, activate 3TP-Lux without TGF-β stimulation. By contrast, RhoB potently antagonizes 3TP-Lux activation and its degradation is inhibited by TGF-β [Engel et al., 1998]. Along with direct binding of c-fos to AP1 elements in the PAI-1 promoter [Yingling et al., 1997], these data implicate AP1 in TGF-β-mediated transcription of the PAI-1 gene. However, transient coexpression of Smad3 and

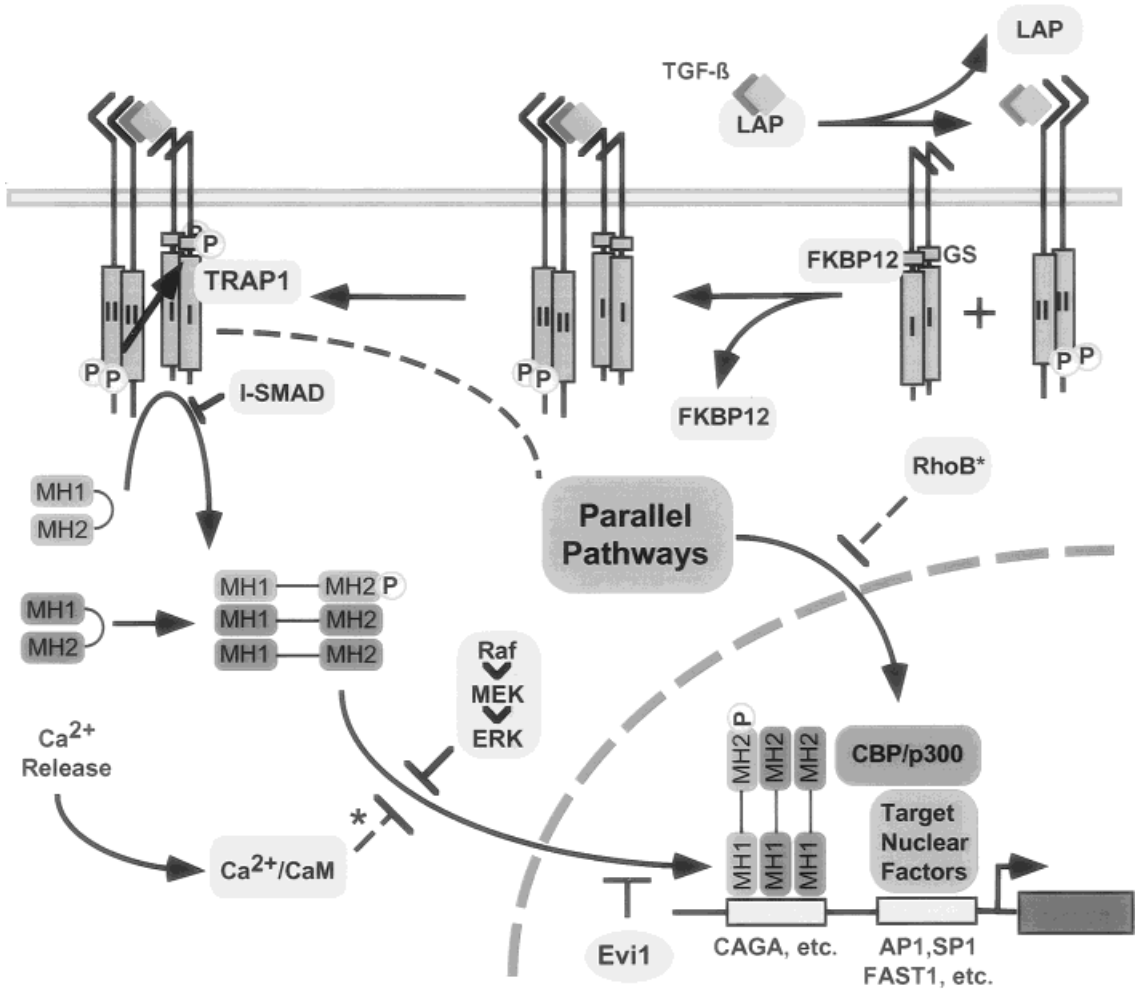

Fig. 3. Transcriptional activation by TGF-β: A cooperative signaling paradigm. In the basal state, TGF-β signaling is subject to extensive negative regulation. The ligand is sequestered in an inactive complex with latency-associated peptide (LAP), while spontaneous receptor activation is antagonized by FKBP12. Pathway-restricted SMADs are negatively regulated by intramolecular interactions between MH1 and MH2 domains. TGF-β mobilizing stimuli allow ligand binding, receptor complex and SMAD activation as described above. Activated SMAD complexes enter the nucleus, and may recognize discrete elements in target promoters. Additionally, stimulation of parallel pathways, such as the JNK pathway, activates ubiquitous transcriptional regulators. Both activated SMADs and ubiquitous transcription factors are recognized by the co-activator CBP/p300, which serves as a bridge to the basal transcription machinery. Additional negative regulation complements that imposed in the basal state. TRAP1 selectively binds activated TβRI and antagonizes transcriptional responses. Inhibitory SMADs (I-SMADs), whose expression is inducible by TGF-β, also block transcriptional responses to TGF-β perhaps by competing with pathway-restricted SMADs for activated TβRI. Evi1 associates with nuclear SMAD complexes to antagonize growth and transcriptional effects of TGF-β. Calmodulin (CaM) binds SMADs in a Ca^{2+}-dependent manner to inhibit transcription. The point of CaM-mediated inhibition is not yet defined, as indicated (*). Nuclear translocation of SMADs is antagonized by ERK-mediated phosphorylation in the linker region. RhoB inhibits transcriptional activation by TGF-β, perhaps by inhibiting parallel pathway signaling. **Color plate on page 320.**

Smad4 also activates 3TP-Lux in the absence of TGF-β [Zhang et al., 1996]. The presence of a SMAD-binding CAGA-box adjacent to the distal AP1 element in the PAI-1 promoter [Dennler et al., 1998] may explain the shared ability of these two pathways to produce TGF-β-specific responses. TGF-β receptor activation may promote simultaneous activation of the SMAD and JNK pathways, which then converge upon the promoter to optimally regulate PAI-1 expression. Two additional CAGA-boxes are found in the PAI-1 promoter, along with less closely linked AP1 elements [Keeton et al., 1991; Dennler et al., 1998], suggesting other potential loci for SMAD/AP1 synergy. Similar cooperative signaling may occur for other TGF-β-

regulated genes. Putative CAGA-boxes are present in the α2(I) procollagen promoter overlapping an essential AP1 site, and near a hexanucleotide FAST-1 binding site in the Mix.2 promoter. However, the CAGA-box is likely not the only SMAD binding element, as neither the $p15^{INK4B}$ nor p21 promoters display such an element near SP1 sites implicated in TGF-β regulation of these genes. Other SMAD binding elements are likely in mammalian promoters, perhaps more closely related to those found in the *vg* quadrant enhancer and the *ubx* promoter. In support of this hypothesis, a functional interaction between SP1 and a Smad3/Smad4 complex was recently described in TGF-β-mediated activation of the p21 promoter [Moustakas and Kardassis, 1998]. Moreover, the ability of the MH2 domain to alone promote transcriptional events implies that protein–protein interactions rather than SMAD DNA binding may be paramount in some circumstances.

In addition to factors that recognize specific *cis*-acting elements, transcriptional activation also requires the recruitment of cofactors that provide a bridge to the basal transcription machinery. In TGF-β signaling, one such bridge appears to be CBP/p300, which interacts with activated Smad2 or Smad3 and potentiates both SMAD and TGF-β-mediated transcriptional responses [Feng et al., 1998; Janknecht et al., 1998]. The SMAD MH2 domain and a 100-amino acid domain in the CBP C-terminus were sufficient for the interaction. CBP is known to interact with several classes of sequence-specific transactivators, including CREB, AP1, Elk-1, STAT-1α, and NF-κB, and additionally binds components of the basal transcription machinery including TFIIB and RNA polymerase II. In addition, CBP displays histone acetyltransferase activity in a central domain, presumably giving it the ability to modify chromatin structure. Notably, a C-terminal fragment of CBP that lacks histone acetyltransferase activity blocked Smad3-dependent transactivation. The involvement of CBP/p300 in TGF-β signaling not only provides a potential link to the basal transcription machinery, but through coincident interactions with other factors, may help coordinate inputs from multiple pathways at a single promoter. Moreover, competition for a limiting pool of CBP/p300 may allow intracellular integration of multiple extracellular stimuli, favoring particular transcriptional outputs at the expense of others.

PARALLEL PATHWAYS AND MALIGNANCY

The significance of parallel pathways in TGF-β signaling is suggested by the dual roles of TGF-β in tumorigenesis. Its growth inhibitory, immunosuppressive, and apoptotic effects give TGF-β qualities of a tumor suppressor. Naturally occurring inactivating mutations in TβRII in human tumors support such a role for TGF-β. However, several groups have described TGF-β-induced morphological changes and invasive characteristics in cells otherwise refractory to TGF-β-mediated growth inhibition. In transgenic mice with keratinocyte-targeted TGF-β expression, outgrowth of benign papillomas was inhibited, consistent with the growth inhibitory role of TGF-β. However, those tumors that escaped growth inhibition by TGF-β manifested a higher rate of malignant conversion, often characterized by an invasive, spindle cell phenotype [Cui et al., 1996]. This phenotype required TGF-β receptor function [Portella et al., 1998], suggesting separation in signal transduction pathways downstream of the receptor complex. Given that SMAD pathway disruption abrogates TGF-β-mediated growth inhibition, the effects of TGF-β on cell morphology and invasiveness may stem from parallel pathway activation, in cooperation with other oncogenes (Fig. 4). The observed inhibition of Smad1 function after ERK activation might represent a regulatory scheme in normal physiology that is constitutively recapitulated by SMAD pathway mutations in Ras transformed cells [Kretzschmar et al., 1997b]. Rho family proteins, which contribute significantly to cell morphology and motility, are attractive candidate components of parallel pathways mobilized by TGF-β exposure (see above). Tumor cells harboring mutations in growth regulatory pathways downstream of the receptor could have enhanced malignant potential, evading growth inhibition by TGF-β yet retaining pro-invasive abilities.

CONCLUSIONS

Knowledge of signaling mechanisms that underlie responses to TGF-β family members has advanced at a rapid pace. Extensive negative regulation seems to be a central theme (Fig. 3), preventing signals at subthreshold ligand concentrations and restricting signaling after path-

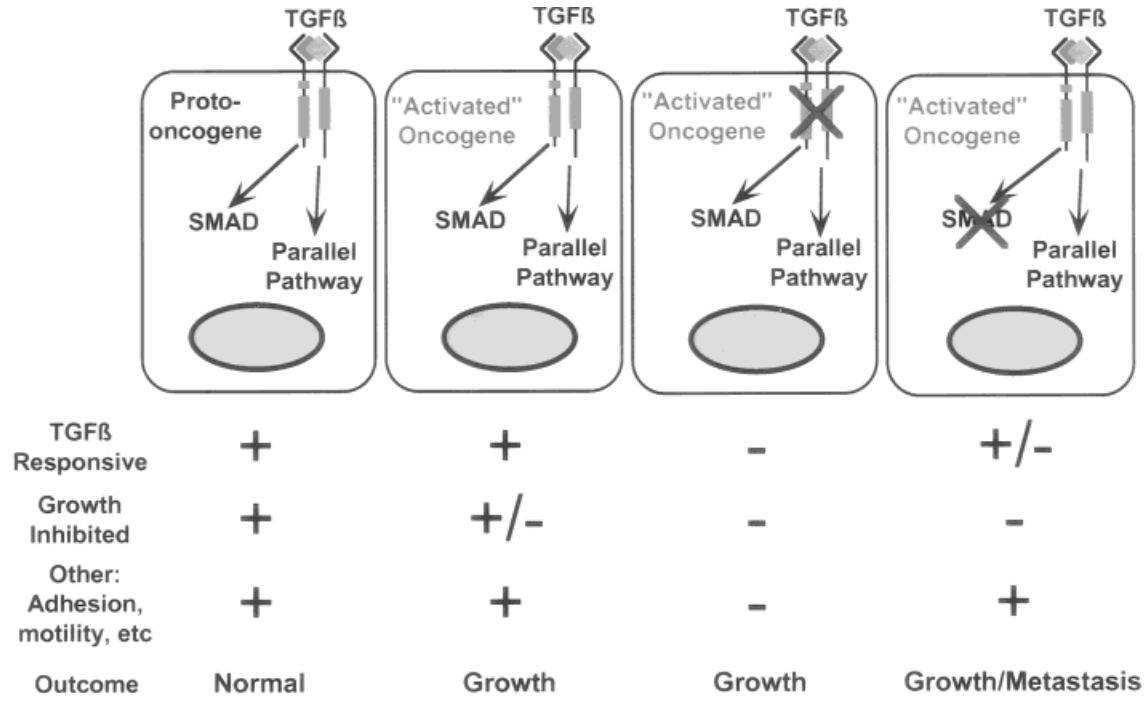

Fig. 4. Proposed effects on transformed cells due to mutations in the TGF-β signaling pathway. Presuming a cooperative signaling paradigm, TGF-β may differentially affect the behavior of transformed cells. Its influence may reflect the position of a mutation in the TGF-β signaling machinery. Mutations at the level of the TGF-β receptor should abolish all responses to TGF-β. However, SMAD mutations may ablate certain effects of TGF-β, such as the growth inhibitory response, while preserving others that are more dependent on parallel pathways. Transformed cells that escape TGF-β-mediated growth inhibition may achieve a growth advantage through effects elicited by parallel signaling pathways. **Color plate on page 321.**

way activation. Given that activation of these pathways has important influences on cell and tissue fate, it would seem that such restraint is warranted. Once activated, transcriptional responses to TGF-β are governed by the cooperative influence of multiple nuclear factors and the pathways that regulate them. Although the SMAD family of proteins is essential, it remains to be determined how they synergize with other nuclear factors to control gene expression. A cooperative model predicts that receptor activation stimulates not only a SMAD pathway, but also parallel pathways that target additional trans-acting factors. Identifying these parallel pathways and determining how they communicate with activated receptors will be areas of intense research. We may also discover that subsets of TGF-β responses occur independent of SMAD signaling. The ability to separate TGF-β-mediated morphological responses from growth regulatory effects may reflect SMAD independent signaling through parallel pathways. In conjunction with signals from other oncogenes, these parallel pathways may profoundly influence the behavior of transformed cells in response to TGF-β. From recent advances, we can now begin to define the signaling requirements for specific responses to TGF-β family members, and from that information develop response-specific agonists and antagonists.

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