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REVIEW

Controlling TGF-β signaling

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The transforming growth factor β (TGF-β) family of hormonally active polypeptides have attracted much attention because of their ability to control cellular functions that underwrite animal embryo development and tissue homeostasis. TGF-β family members act by modifying the expression of specific sets of target genes, and biologists pursuing the elucidation of TGF-β signaling mechanisms have turned up a fairly simple system, linking membrane TGF-β receptors to such genes (for recent reviews, see Heldin et al. 1997; Massagué 1998; Whitman 1998; Massagué and Wotton 2000). If a TGF-β signaling system can be so simple, and yet so powerful, then an elaborate network of regulators must keep control over the inputs, activity, and outcomes of this system. A multitude of regulatory mechanisms have been recently uncovered that control the access of TGF-β family members to their receptors, the activity of their receptors and receptor substrates, and the nuclear function of the transcriptional complexes generated by this pathway. The regulatory mechanisms operating in the prereceptor phase of a TGF-β signaling pathway can be as intricate and physiologically important as those operating downstream of TGF-β receptors. These control mechanisms, which are central to understanding the physiology of TGF-β signaling, are reviewed here.

Signal transduction

A simple signaling engine for a large family of agonists

The bone morphogenetic proteins (BMPs) form the largest group within the TGF-β family and include BMP2, BMP7, and growth and differentiation factor-5 (GFD5), additional closely related vertebrate factors, and the *Drosophila* orthologs decapentaplegic (Dpp) and 60A (for reviews on the TGF-β family, see Gaddy-Kurten et al. 1995; Hogan 1996; Mehler et al. 1997; Letterio and Roberts 1998; Massagué 1998; Schier and Shen 2000). The BMPs are known for their remarkable roles as instructive signals during embryogenesis, and in the maintenance and repair of bone and other tissues in the adult. Nodal and related factors form a separate, structurally more divergent, group also with important roles in embryogenesis. The factors in this group account for the "Activin-like" signals whose role in laying out the body plan and other aspects of embryogenesis is complementary to that of the BMPs. The various forms of TGF-β and Activin are structurally further removed from the BMPs, and are best known for their roles in late stages of embryogenesis and in the mature organism. The TGF-βs are critical inhibitors of epithelial growth and immune and hematopoietic functions, as well as strong promoters of connective tissue growth among many other functions. The Activins are important players in the mammalian endocrine reproductive axis. Several distant members, most prominently the anti-Müllerian hormone (AMH, also known as MIS), complete the TGF-β family.

For all of the diversity and physiological importance of the responses that this family can elicit, a disarmingly simple system lies at the core of its signaling pathways in vertebrates, insects, and nematodes. The basic signaling engine consists of two receptor serine/threonine protein kinases (receptor types I and II) and a family of receptor substrates (the Smad proteins) that move into the nucleus. The ligand assembles a receptor complex that activates Smads, and the Smads assemble multisubunit complexes that regulate transcription (Fig. 1; for review, see Massagué 1998). Two general steps thus suffice to carry the TGF-β stimulus to target genes.

A centerpiece of this engine is the type I receptor. In the basal state, a wedge-shaped structure, the GS region, of this receptor (named after a characteristic SGSGSG sequence that it contains) presses against the kinase domain, dislocating its catalytic center (Fig. 2; Huse et al. 1999). When brought into the complex by the ligand, the type II receptor phosphorylates the GS region, resulting in the activation of the receptor I kinase. This kinase then phosphorylates Smad proteins which, to date, are the only direct substrates with demonstrated ability to mediate gene responses to the TGF-β family.

In vertebrates, the type I receptors for TGF-β, Activin and Nodal, recognize Smad2 and Smad3, whereas the BMP and GDF receptors recognize Smad1, Smad5, and Smad8 (Fig. 1). Parallel systems have been identified in *Drosophila* and *Caenorhabditis elegans* (Padgett et al. 1998; Raftery and Sutherland 1999, and references therein). Receptor-mediated phosphorylation of this group of regulated Smads (which are referred to as R-Smads) occurs in the carboxy-terminal sequence SSxS (Fig. 3) and allows the R-Smads to accumulate in the

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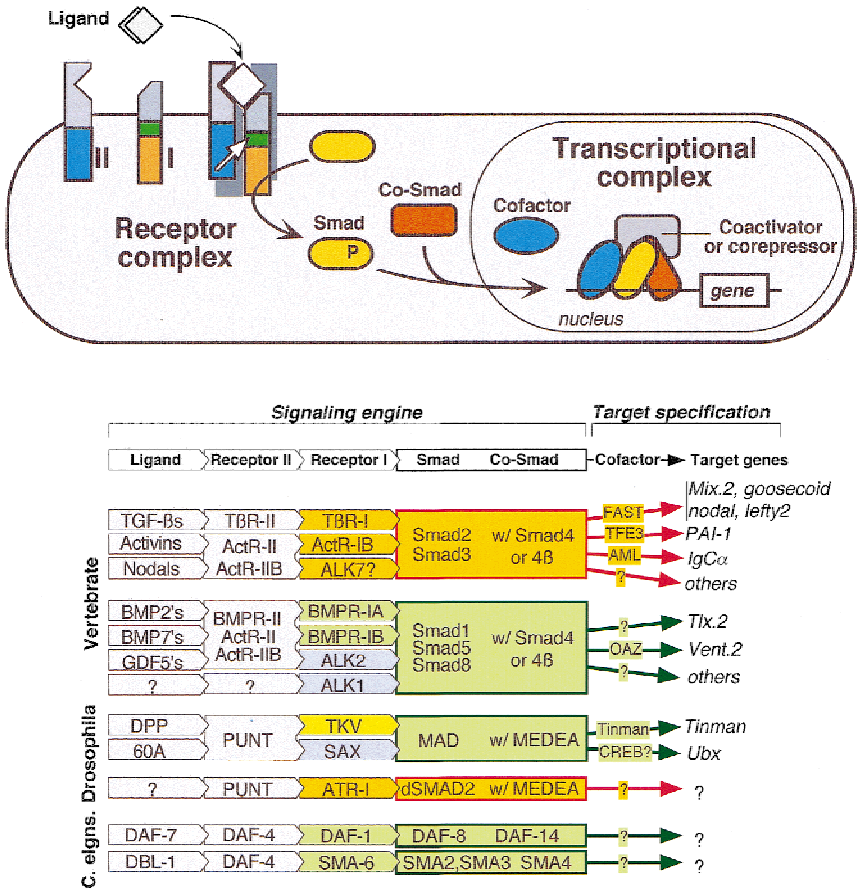

Figure 1. TGF-β signaling via Smads: Converging in and branching out of a simple signaling engine. (*Top*) The basic signaling engine: The ligand assembles a receptor complex that phosphorylates Smads, and the Smads assemble a transcriptional complex that regulates target genes. The type II receptors are activators of the type I receptor. Smads are direct substrates of type I receptors. The assembly of receptor-phosphorylated Smads with co-Smads is essential for many transcriptional responses. Smads gain access to target genes by synergistically binding to DNA with cell-specific cofactors, many of which remain unknown. The Smad complex can recruit coactivators or corepressors that determine the outcome. (*Bottom*) Variegation, convergence, and then, branching. Two subfamilies of type I receptors (orange and green) recognize each subfamily of Smads. All R-Smads share the same co-Smads. Analogous TGF-β signaling pathway relationships exist in *Drosophila* and *C. elegans*. TβR-I, ActR-IB, BMPR-IA, and BMPR-IB are also known as ALK5, ALK4, Alk3, and ALK6, respectively.

nucleus. On their way to the nucleus, the activated R-Smads associate with the related proteins Smad4 or Smad4β in vertebrates [Smad4β has been described to date only in *Xenopus*; (Howell et al. 1999; Masuyama et al. 1999)] and Medea in *Drosophila*. This second group, referred to as the co-Smads, are not receptor substrates, but their presence is required for many of the gene responses induced by Smads.

The R-Smads and the co-Smads consist of conserved amino- and carboxy-terminal domains that form globular structures (Fig. 3) (Shi et al. 1997, 1998). Between these two domains lies a linker region that is full of regulatory sites (see below). The amino-terminal MH1 domain has DNA-binding activity (except in the major splice form of Smad2, which contains an insert that prevents DNA binding), whereas the carboxy-terminal MH2 has transcriptional activity. Receptor-mediated phosphorylation appears to relieve these two domains from a mutually inhibitory interaction. The L3 loop and the α helix-1 (αH-1) in the MH2 domain of a Smad (Fig. 3; Lo et al. 1998; Chen and Massagué 1999) and the L45 loop in the kinase domain of a type I receptor (Feng and Derynck 1997; Chen et al. 1998) specify the Smad–receptor interaction (Fig. 2), whereas the αH-2 specifies interactions with certain DNA-binding cofactors (Chen et al. 1998). A highly basic surface patch conserved around the L3 loop of all R-Smads, but not present in Smad4 (Wu et al. 2000), and a complementary surface pattern on the TGF-β type I receptor (TβR-I) kinase domain (Huse et al. 1999) may also be important in receptor–Smad recognition.

Target specification

How can such a simple system mediate a variety of cell-specific gene responses? The principal Smads in the TGF-β/Activin/Nodal pathways lead to target genes different from those controlled by the Smads in the BMP pathways. Although the choice of Smad by a given TGF-β family receptor provides a first level of target gene specification, a given Smad can lead to radically different responses depending on the cell type. The genes recognized by a Smad complex in a given cell will determine the final response of that cell to the Smad-dependent agonist.

The choice of target genes by an activated Smad complex is made by the association of this complex with specific DNA-binding cofactors (Fig. 1; for review, see Massagué and Wotton 2000). The MH1 domain interaction with DNA is not selective: Smads in the TGF-β/Activin/Nodal pathways and in the BMP pathways all recognize the same sequence, CAGAC (Shi et al. 1998). However, this interaction is of low affinity, which means that DNA-binding cofactors must be involved to

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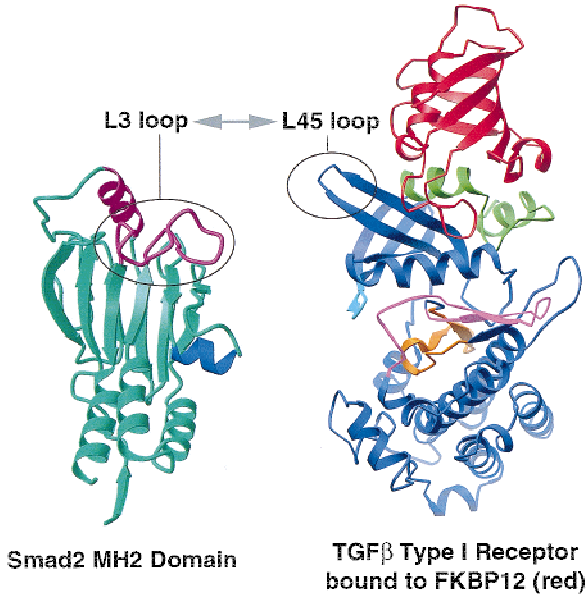

Figure 2. TGF-β receptor regulation and interaction. The type I receptor in the basal state is maintained inactive by the GS domain (green), which presses against and dislocates the catalytic center of the kinase domain (blue). The immunophilin FKBP12 (red) binds to the GS domain, occluding its phosphorylation sites. Phosphorylation of the GS domain by the type II receptor in the ligand-induced complex is predicted to remove the inhibitory constraint. The specificity of receptor–Smad recognition is dictated by the L45 loop region on the receptor and the L3 loop region on the MH2 domain of Smad (see also Fig. 3). [Adapted from Shi et al. (1997) and Huse et al. (1999).]

provide a tight and highly specific recognition of regulatory elements in target genes. Several such cofactors have been identified (Fig. 1), including the DNA-binding proteins FAST (X. Chen et al. 1997; Labbé et al. 1998; Saijoh et al. 2000), OAZ (Hata et al. 2000), and Mixer and Milk (Germain et al. 2000), which have no intrinsic transactivating activity, and the previously known transcription factors AP-1 (Jun–Fos) (Zhang et al. 1998; Wong et al. 1999), TFE3 (Hua et al. 1999), and AML proteins (Hanai et al. 1999; Pardali et al. 2000) that function independently of Smads in other contexts.

Once a Smad complex binds to DNA it may control the transcription of target genes by altering nucleosome structure, thereby remodeling the chromatin template. Via the MH2 domain, Smads can bind the coactivators p300/CBP, which have histone acetyl transferase activity, and the corepressors TGIF, c-Ski and SnoN, which recruit histone deacetylases (for review, see Derynck et al. 1998; Massagué and Wotton 2000). The transcriptional activity of Smad MH2 domain is manifest in fusions to the Gal4p DNA-binding domain, and requires the presence of a co-Smad. Smads and co-Smads may jointly recruit the necessary set of coactivators or corepressors to orchestrate a transcriptional response. Beyond this, little is currently know about the transcriptional events that are activated by a Smad complex on DNA.

Variegation, convergence, and branching

Given the diversity of responses induced by the TGF-β family members, it may be surprising that a multitude of factors in this family converge on a handful of receptors which, in turn, funnel the signaling through an even smaller number (so far) of Smad proteins (see Fig. 1). Beyond the Smads, the signaling processes branch out toward different outcomes, through the agency of specific DNA-binding cofactors, coactivators, and corepressors. Differences in the kinetics and mode of interaction of the different ligands with the receptors, the different receptors with Smads, and the different Smads with target genes establish functionally important—if biochemically discrete—distinctions between the various components of the basic TGF-β signaling engine. Factors controlling these protein–protein and protein–DNA interactions have an enormous impact on the biological outcome.

Controlling the ligands

The activity of TGF-β factors is modulated by various families of diffusible ligand-binding proteins (Fig. 4). These proteins prevent ligand access to the signaling receptors. As such, these proteins may contribute to the formation of morphogen gradients during embryogenesis, to the relay of signals by extracellular signal transduction pathways, and to the homeostasis of signaling inputs in a tissue. However, the structural diversity and complexity of some of these ligand-binding proteins raises the possibility that they may have other roles, such as serving as growth factor reservoirs, or as "pillboxes" for the concerted delivery of different growth factors at once.

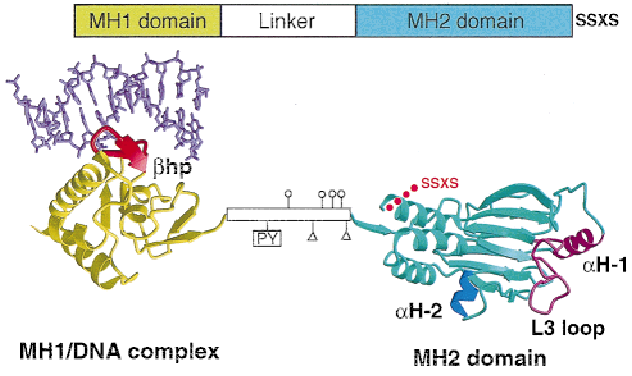

Figure 3. Smad domains. The conserved MH1 and MH2 domains of R-Smads form globular structures with surface protrusions and pockets for interactions with the DNA sequence CAGAC (purple) via the β hairpin (βhp), with the type I receptor via the L3 loop and secondarily the α-helix 2 (αH-2), and with DNA-binding cofactors such as FAST via αH-1 in R-Smads. The linker region, of unknown structure, contains phosphorylation sites for Erk MAPKs (○), consensus sites for calcium-regulated kinases (△), and one PY motif for recognition by WW domains (see text for details). Receptor-mediated phosphorylation occurs at the carboxy-terminal SSxS motif. [Adapted from Shi et al. (1997, 1998).]

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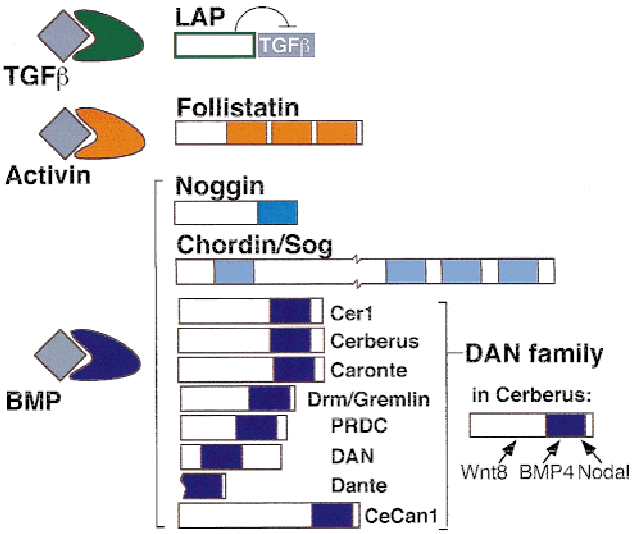

Figure 4. Binding proteins that prevent ligand access to signaling receptors. LAP is the cleaved propeptide from the TGF-β precursor; it remains noncovalently associated with TGF-β. Follistatin is an Activin antagonist that can also recognize BMPs (not shown). Noggin and Chordin are structurally unrelated to the DAN family members, but all three groups act as BMP antagonists. Colored boxes represent cysteine-rich regions. Cerberus can bind, through separate regions, Wnt8 and Nodal in addition to BMP4.

Latent TGF-β and its intricate activation process

TGF-β is synthesized as a prohormone that is cleaved in the secretory pathway into an amino-terminal propeptide and a carboxy-terminal fragment that constitutes the mature growth factor (Fig. 4). Unlike most other hormones, the mature TGF-β remains noncovalently associated with its propeptide after secretion (for review, see Roberts and Sporn 1990). Mature TGF-β in this complex is not recognized by the signaling receptors; hence, the term latency-associated protein (LAP) designates the TGF-β propeptide. A family of large secretory glycoproteins known as latent TGF-β-binding proteins (LTBPs) covalently bind to LAP via disulfide bonds. LTBPs are not required for maintenance of TGF-β latency but may instead facilitate the secretion, storage, or activation of the TGF-β–LAP complex.

The physiological activation process of latent TGF-β is currently understood only in part, but it seems clear that this is a multistep process. Many different components including the plasminogen activation cascade, thrombospondin, and the mannose 6-phosphate receptor have been suggested to be involved in this process (Taipale et al. 1994; Nunes et al. 1997; Rifkin et al. 1997), but recent genetic evidence points at thrombospondin-1 (TSP-1) and the cell adhesion receptor αvβ6 integrin as important participants in this process in vivo. TSP-1, a large homotrimeric protein secreted by many cell types, can activate latent TGF-β in vitro through a conformational modification of LAP and appears to be responsible for a significant proportion of the activation of TGF-β1 in vivo (Crawford et al. 1998). *TGF-β1* null mice phenocopy *TSP-1* null mice, and systemic treatment with a peptide that blocks TGF-β1 activation by TSP-1 causes lung and pancreas alterations similar to those of *TGF-β1* null animals. A TSP-1 peptide that activates latent TGF-β1 reverses these lung and pancreatic abnormalities. In separate studies, the TGF-β1–LAP complex has been shown to be a ligand for the integrin αvβ6. αvβ6-expressing cells may induce spatially restricted activation of TGF-β1, providing an explanation for the propensity to inflammation in mice lacking this integrin (Munger et al. 1999). A different type of protease, matrix metalloproteinase-2 and -9, which are implicated in tumor invasion and angiogenesis as cell surface-bound proteases, have also been shown to activate latent TGF-β (Yu and Stamenkovic 2000).

Activin control by Follistatin

Activin was originally identified as an inducer of follicle-stimulating hormone (FSH) from the pituitary and has a central role in the regulation of the reproductive axis (Gaddy-Kurten et al. 1995). Follistatin is a soluble secreted glycoprotein that suppresses the release of FSH by binding to Activin and inhibiting its interaction with Activin receptors (de Winter et al. 1996). Follistatin can also bind to BMPs, with similar effects (Iemura et al. 1998) and has been shown to induce neural tissue in *Xenopus* embryonic explants, probably by blocking BMP activity (Hemmati-Brivanlou et al. 1994). As many of the other TGF-β family-binding proteins discussed below, Follistatin contains cysteine-rich modules of a type also found in osteonectin, agrin, and other extracellular matrix glycoproteins. These modules may constitute growth factor-binding regions (Fig. 4).

The importance of Follistatin in modulating Activin activity is evident in *follistatin*-deficient mice, which exhibit abnormal whisker and tooth development and hard-palate defects (Matzuk et al. 1995b). Defects in development of these organs were also observed in *Activin A*-deficient mice (Matzuk et al. 1995a). *follistatin*-deficient mice also have defects that are not observed in *Activin* mutant mice, consistent with a role for Follistatin in regulating other factors. Follistatin is produced and localized to prostate tissue from men with high grade cancer, where it has been proposed to bind to autocrine Activin and inhibit its antiproliferative activity (McPherson et al. 1999).

BMP antagonists and their roles in embryogenesis

Noggin and Chordin

The dorsal lip of the amphibian gastrula embryo, also called the Spermann's organizer (SO), promotes formation of dorsal tissues within the mesoderm and induces neural tissue in animal cap ectoderm that would otherwise become epidermis. Chordin and Noggin are secreted proteins expressed in the SO. Both can induce neural markers in the ectoderm and convert ventral me-

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soderm to muscle (a dorsal tissue) in explants of gastrula ventral marginal zone (Piccolo et al. 1996; Zimmerman et al. 1996). Noggin is a small glycoprotein (32 kD) produced as a homodimer, whereas Chordin is a large protein (120 kD). Noggin contains a carboxy-terminal cysteine-rich domain (Fig. 4). Chordin contains cysteine-rich repeats similar to those found in TSP-1, procollagens I and III, and von Willebrand factor. Although not structurally related, both Chordin and Noggin bind specifically to BMPs, but not to Activin or TGF-β, and antagonize BMP signaling by blocking BMP interaction with cell-surface receptors (Piccolo et al. 1996; Zimmerman et al. 1996). Noggin can also bind to and inhibit *Xenopus* GDF6, preventing its ability to induce epidermis and block neural tissue formation (Chang and Hemmati-Brivanlou 1999). The *Drosophila short gastrulation* gene product, Sog, is a structural and functional homolog of Chordin that has been proposed to form an inhibitory complex with either Dpp or the related ligand Screw and interferes with binding to Dpp receptors (Holley et al. 1996).

In mice, *Noggin* is expressed in the node, notochord, dorsal somite, condensing cartilage, and immature chondrocytes and is required for patterning of the neural tube and somites (Brunet et al. 1998; McMahon et al. 1998). Antagonism of BMP activity by Noggin is critical for proper skeletal development: Noggin-null mice had excess cartilage and failed to initiate joint formation (Brunet et al. 1998; McMahon et al. 1998). The function of Noggin in joint formation is further manifested by the identification of dominant mutations in *Noggin* in two human genetic disorders: proximal symphalangism and multiple synostoses syndrome (Gong et al. 1999). Both disorders are characterized by bony fusions of joints. *Noggin* is also expressed in the follicular mesenchyme, where it neutralizes the inhibitory action of BMP4 on hair-follicle induction (Botchkarev et al. 1999). Noggin expression in chondrocyte and osteoblast cultures is increased by BMP signaling, suggesting that Noggin may participate in a BMP negative feedback loop (Gazzerro et al. 1998; Kameda et al. 1999).

The DAN family

The DAN family of vertebrate BMP antagonists includes mammalian DAN (Stanley et al. 1998a), Dante (Pearce et al. 1999), Drm/Gremlin (Hsu et al. 1998; Stanley et al. 1998a), Cer1 (Stanley et al. 1998b; Simpson et al. 1999), and protein related to DAN and cerberus (PRDC) (Pearce et al. 1999), *Xenopus* Cerberus (Piccolo et al. 1999), chick Caronte (Rodriguez-Esteban et al. 1999; Yokouchi et al. 1999), and *C. elegans* CeCan1 (Pearce et al. 1999). Like Noggin and Chordin, this family of BMP antagonists is thought to bind BMPs, preventing their interaction with the signaling receptors, as biochemically confirmed in various cases (Hsu et al. 1998; Piccolo et al. 1999; Yokouchi et al. 1999). The region of highest similarity among Cerberus/DAN proteins is a 90-amino-acid cysteine-rich region. This region is related to the "cystine knot," a motif that is present in the TGF-β family and other secretory polypeptides and forms an extended three-dimensional structure strongly stabilized by three interlocking disulfide bonds (McDonald and Hendrickson 1993). Proteins that contain this motif often form disulfide-linked homodimers, and this may also be the case in the Cerberus/DAN family (Pearce et al. 1999). The BMP-binding region in Cerberus and Caronte includes the cysteine-rich domain (Piccolo et al. 1999; Yokouchi et al. 1999) and has been suggested to bind as an extended surface to the BMP monomer (Rodriguez-Esteban et al. 1999).

Unlike Cerberus, Caronte, Gremlin, and other members of this family that were identified as regulators of developmental processes, *Dan* was initially identified as a gene whose expression is significantly reduced in a variety of transformed rat fibroblasts, including v-*src*-, SV40- and v-*mos*-transformed cells, compared to untransformed controls (Enomoto et al. 1994). *Dan* overexpression can inhibit the tumorigenic activity of *src*-transformed fibroblasts (Enomoto et al. 1994). Similar properties have been described for DRM, a rat homolog of Gremlin (Topol et al. 1997). How DAN and DRM exert these effects remains unknown.

Cerberus, a multivalent antagonist of signaling pathways

cerberus was isolated in a search for transcripts that are concentrated in the SO of the *Xenopus* early embryo (Bouwmeester et al. 1996). Microinjection of *cerberus* mRNA into *Xenopus* embryos has the extraordinary ability to induce ectopic heads, neuralize the ectoderm, duplicate heart and liver, and suppress the trunk–tail mesoderm. Cerberus is a high-affinity BMP4-binding protein, and some of its effects are mediated by its ability to block BMP (Piccolo et al. 1999). However, Cerberus also binds the mesoderm-inducing factor Xnr1 (Piccolo et al. 1999) and the inducer of secondary axis Xwnt8 (Glinka et al. 1997; Piccolo et al. 1999). These factors seem to bind to separate sites in Cerberus: Xnr1 and BMP4 bind in the cystine-knot region, whereas Xwnt8 binds to the unique amino-terminal half of Cerberus. Thus, Cerberus appears to restrict trunk formation to the posterior part of the body by coordinately antagonizing three trunk-forming pathways—the BMP, Nodal and Wnt pathways—in the anterior part.

Signal relay by Caronte and Gremlin

Studies on these two members of the Caronte/DAN family have illustrated their fascinating roles as BMP antagonists in the relay of developmental signals and the control of the BMP–Nodal counterbalancing system. *caronte* was identified in the context of studies on the establishment and expansion of vertebrate left–right asymmetry domains (Rodriguez-Esteban et al. 1999; Yokouchi et al. 1999). During chick embryogenesis, bilateral symmetry appears to be initially broken around the chick organizer, Hensen's node, possibly by the unidi-

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rectional rotation of cilia (for review, see Vogan and Tabin 1999). An Activin-like factor, probably Nodal, acting on the incipient right side, then induces *fibroblast growth factor-8* (*Fgf8*), and limits *Sonic hedgehog* (*Shh*) expression to the left side (Rodriguez-Esteban et al. 1999; Yokouchi et al. 1999, and references therein). By embryonic stage 4 these events have created a small asymmetric domain of signaling molecules straddling the midline, with Shh dominating on the left and FGF8 on the right (Fig. 5A).

By embryonic stage 7, the asymmetry has expanded to a larger area encompassing the lateral plate mesoderm (LPM) on both sides of the midline. Various BMPs (*Bmp2*, *Bmp4*, and *Bmp7*) are expressed along the midline and throughout the LMP but can signal only on the right side, where they suppress *nodal* expression. On the left side, Shh inhibits BMP signaling, allowing the expression of *nodal* and its dominance over the left LPM. Shh is thought to act locally at its site of production near the node because it is a lipophilic, poorly diffusible factor. However, Shh induces Caronte, which is diffusible and relays the signal throughout the left LPM, antagonizing BMPs and allowing *nodal* expression (Rodriguez-Esteban et al. 1999; Yokouchi et al. 1999).

What prevents Caronte and Nodal from spilling over to the right side? It appears that Caronte allows the expression of an additional TGF-β family member, *Lefty1*, at the midline. It has been proposed that Lefty1 may bind *Caronte* at the border, preventing the contralateral spread of asymmetric signals (Yokouchi et al. 1999) (Fig. 5A). Similar events take place in the mouse, where it has been shown that Nodal signal transduction maintains *nodal* expression and induces *lefty2* through a Smad/FAST-mediated activation of a left-side-specific enhancer of these two genes (Saijoh et al. 2000). Lefty2 acts as a Nodal antagonist, possibly by inhibiting binding to the receptor ActR-II, and sets a limit to the territory of Nodal action (Bisgrove et al. 1999; Meno et al. 1999; for review, see Schier and Shen 2000).

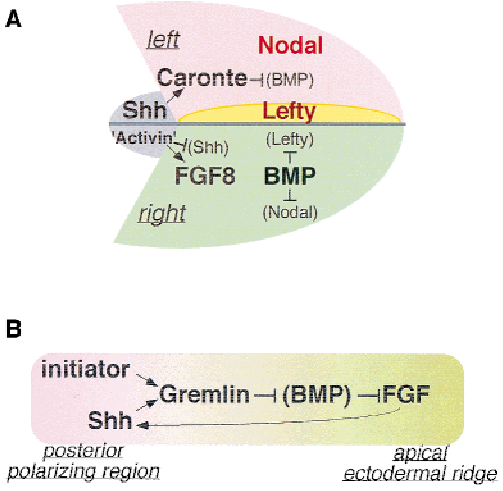

Figure 5. Relaying signals via BMP inhibitors. Caronte and Gremlin, produced in response to Sonic hedgehog (Shh) signals, inhibit BMPs, thus allowing the expression of Nodal on the left plate mesoderm of the developing chick embryo in the establishment of left–right asymmetry (*A*) or FGF in the apical ectodermal ridge of the developing chick limb bud (*B*). [Adapted from Yokouchi et al. (1999) and Zúñiga et al. (1999), respectively.]

gremlin was isolated in studies to identify dorsalizing factors that can induce a secondary axis in the *Xenopus* embryo (Hsu et al. 1998). Gremlin may also have a role in neural crest induction and patterning, as its expression starts at the tailbud stage and is correlated with neural crest lineages. More recently, however, Gremlin has been shown to be a central player in the reciprocal interactions between the posterior mesenchyme (polarizing region) and a specialized ectodermal structure, the apical ectodermal ridge (AER) during the outgrowth and patterning of the vertebrate limb (Zúñiga et al. 1999). A feedback loop exists whereby SHH signaling by the polarizing region modulates FGF4 signaling by the posterior AER, which in turn maintains the polarizing region (Fig. 5B). An unknown initiator activity induces *Gremlin*, which inhibits BMP signals, allowing *Fgf4* expression. FGF4 then induces SHH, which actively maintains *Gremlin* expression. Thus, the BMP antagonist Gremlin relays the initial signal from the polarizing region to the AER, inducing Fgf4 and establishing the SHH/FGF4 feedback loop.

Antagonists of antagonists shaping morphogenic gradients: the case of Tolloid and Sog

The morphogen hypothesis, which postulates that a gradient of instructive signal can specify multiple cell fates over a range of concentrations, is of major interest in embryology. The Activins and BMPs can specify multiple cell fates over a range of concentrations in vitro, and there is strong but indirect evidence that these factors and Dpp function in this manner in vivo (for review, see Whitman 1998; Dale and Jones 1999; McDowell and Gurdon 1999). However, it has been difficult to visualize gradients of these factors in situ. There is also evidence that a gradient of biologically active factors in a flat field of BMP or Dpp can be established by the presence of gradients of the BMP antagonist Noggin, Chordin, or Sog (Marques et al. 1997; Jones and Smith 1998; for review, see Thomsen 1997; Smith 1999).

The complexity of the mechanisms that can contribute to BMP gradient formation is further compounded by the role of antagonists of BMP antagonists. The *Drosophila* gene product Tolloid and its orthologs in *Xenopus* (Xolloid) and human (BMP1 and hTld1) encode secreted metalloproteases that interact genetically and physically with BMPs (Finelli et al. 1994; Takahara et al. 1994). Xolloid has been shown to cleave Chordin at two specific sites, rendering it unable to antagonize BMP activity (Piccolo et al. 1997). Xolloid also cleaves Chordin in Chordin/BMP inactive complexes, releasing biologically active BMPs from these complexes. Xolloid in vivo specifically interferes with the anti-BMP action of chor-

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din, but not Noggin or Follistatin. A similar role has been proposed for Tolloid in zebrafish (Blader et al. 1997).

Tolloid and Sog are required to shape a gradient of Dpp activity that subdivides the dorsal ectoderm of the *Drosophila* embryo into amnioserosa and dorsal epidermis (Ashe and Levine 1999). Sog-bound Dpp is processed by the protease Tolloid. Paradoxically, Sog appears to be required for amnioserosa formation, which is specified by peak Dpp signaling activity. Localized expression of ectopic *sog* redistributes Dpp signaling in a mutant background in which *dpp* is expressed throughout the embryo. Interestingly, *sog* expression is required not only to diminish Dpp activity near the source of Sog but also to generate peak Dpp signaling far from this source. The long-distance stimulation of Dpp activity by Sog requires Tolloid, whereas Sog-mediated inhibition of proximal Dpp does not. These observations reveal an unusual strategy for generating a gradient threshold of Dpp activity.

In a different setting, namely, the wing imaginal disc, a gradient of Dpp signaling activity is shaped by inputs from the Hedgehog (Hh) pathway. Hh signaling represses the expression of the Dpp receptor *thickveins* at the source of Dpp, limiting the ability of Dpp to activated the Smad pathway in that area (Tanimoto et al. 2000).

Regulating receptor activity

The existence of accessory receptors that promote ligand access to TGF-β signaling receptors and factors that inhibit receptor activation have been known for some time. However, several new developments illustrate the importance of these regulators of TGF-β signaling.

The accessory receptors Betaglycan and Endoglin

Betaglycan (also referred to as type III TGF-β receptor) is a membrane-anchored proteoglycan whose core protein binds with high-affinity TGF-β1, TGF-β2, and TGF-β3 (for review, see Massagué 1998). The heparan sulfate and chondroitin sulfate chains of Betaglycan do not appear to have a role in TGF-β binding or interaction with signaling receptors. Betaglycan lacks a recognizable signaling domain but can facilitate TGF-β binding to the signaling receptors. This function is most apparent with TGF-β2. TGF-β2 on its own has low affinity for the type I and type II signaling receptors, compared to TGF-β1 and TGF-β3. Therefore, cells that express these receptors but lack Betaglycan are poorly responsive to TGF-β2. Examples include some types of endothelial cells, skeletal muscle myoblasts, and hematopoietic progenitor cells. Enforced expression of Betaglycan in these cells augments the binding of TGF-β2 to the signaling receptors, equalizing the sensitivity of the cells to all three forms of TGF-β.

An essential, nonredundant role of Betaglycan in TGF-β signaling was demonstrated recently in the transformation of endothelial progenitors into endocardial cells in the heart (C.B. Brown et al. 1999). Endothelial cells in the cardiac primordium that undergo this epithelial–mesenchymal transformation express Betaglycan. Anti-Betaglycan antisera inhibits this transformation, whereas the misexpression of Betaglycan in nontransforming endothelial cells of the ventricular region allowed the transformation of these cells in response to TGF-β2. The territory of Betaglycan expression in the cardiac endothelium appears to define the prospective endocardium by making endothelial cells competent to respond to TGF-β2.

Another member of this family is Endoglin, a glycoprotein with regions of sequence similarity to Betaglycan, but not a proteoglycan (for review, see Massagué 1998). *endoglin* is expressed at particularly high levels in endothelial cells (hence its name). Mutations in *endoglin* and the orphan type I receptor *ALK1* give rise to similar forms of hereditary hemorrhagic telangiectasia, a disease characterized by bleeding from malformed vessels (Marchuk 1998). Therefore, Endoglin may be the accessory receptor for the ligand of ALK1. The identity of the physiological ligand for Endoglin remains an open question. Endoglin and ALK1 can bind TGF-β (Cheifetz et al. 1992; Attisano et al. 1993), and *endoglin* is required for extraembryonic angiogenesis and heart development in the mouse, as is the case with *TGF-β1* or *TβR-II* (TGF-β receptor type II) (Pece-Barbara et al. 1999). However, the binding of TGF-β to either Endoglin or ALK1 is weak (Cheifetz et al. 1992; Attisano et al. 1993), and *endoglin* overexpression inhibits, rather than enhances, TGF-β responsiveness in cell culture (Letamendia et al. 1998).

FKBP12 as a guardian of type I receptors

FKBP12 is a ubiquitous, highly conserved cytosolic protein, and the target of the immunosuppressive macrolide drugs FK506 and rapamycin. The FK506–FKBP12 complex and the rapamycin–FKBP12 complex bind to and inhibit the protein phosphatase calcineurin and the kinase FRAP/RAFT, respectively (Choi et al. 1996; Crabtree 1999; Sabatini et al. 1999). Physiological targets of FKBP12 in the absence of these agents include cardiac muscle calcium release channels (ryanodine receptors) (Marks 1996; Marx et al. 1998) and inositol triphosphate receptors (Snyder et al. 1998), whose functions are enhanced by FKBP12, and the TGF-β family type I receptors, whose functions FKBP12 inhibits.

The inhibitory effect of FKBP12 on TGF-β receptors (Wang et al. 1996) is caused by the binding of FKBP12 to the GS domain, blocking the phosphorylation of the activation sites by TβR-II (Y. Chen et al. 1997). FKBP12 binds directly to the GS domain and sits on TβR-I like a cap occluding the approach of a kinase to the GS domain phosphorylation sites (Fig. 2; Huse et al. 1999). A TβR-I mutant defective in FKBP12 binding displays increased basal activity, but in the presence of saturating ligand this mutant receptor is not more active than the wild-type receptor (Y. Chen et al. 1997). Ligand-induced assembly of the receptor complex is thought to cause the release of FKBP12, allowing receptor activation (Stock-

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well and Schreiber 1998). Binding involves the same hydrophobic pocket of FKBP12 that binds macrolide drugs, which is consistent with the fact that FK506, rapamycin, and their derivatives can cause FKBP12 dissociation from TβR-I and consequently relieve the inhibitory effects of FKBP12 on TGF-β signaling.

These observations suggest that FKBP12 binding to type I receptors in the basal state may serve to prevent leaky activation of these receptors by ligand-independent encounters with type II receptors or other protein kinases. This model has been questioned on the grounds that *FKBP12* null mice do not phenocopy TGF-β family gain of function mutations, and cells derived from these animals have no apparent differences in TGF-β signaling compared to wild-type counterparts (Bassing et al. 1998; Shou et al. 1998). However, the widely distributed close structural and functional homolog, FKBP12.6, also interacts with the TGF-β type I receptor (Datta et al. 1998), suggesting that the absence of TGF-β gain of function in *FKBP12* null animals is due to the redundant role of FKBP12.6 or other FKBP12 family members.

Negative BMP feedback by the pseudoreceptor BAMBI

BAMBI (BMP and activin membrane-bound inhibitor) was identified as an inhibitor of BMP signaling during *Xenopus* embryo development (Onichtchouk et al. 1999). BAMBI is a transmembrane protein whose extracellular domain has sequence similarity to TGF-β type I receptors. BAMBI can become incorporated into ligand-induced complexes with type I receptors, but it primarily hinders signaling by forming heterodimers with type I receptors and interfering with their activation (Fig. 6; Onichtchouk et al. 1999). BAMBI also inhibits signaling by type I receptors with constitutively activating mutations in the GS domain. The short intracellular domain of BAMBI has limited sequence similarity to the E6 loop and catalytic loop of the type I receptors. The E6 loop is involved in homodimeric contacts (Huse et al. 1999), and these contacts are important for receptor kinase activation (Weis-Garcia and Massagué 1996).

BAMBI can potently inhibit signaling by most members of the type I receptor family except ALK2, and in *Xenopus* embryos ectopic overexpression of BAMBI inhibits both Activin-like and BMP-like signals. However, during *Xenopus* embryogenesis endogenous BAMBI functions as a negative feedback loop in BMP signaling: Its expression pattern closely matches that of *Bmp4*, and maintenance of *Bambi* expression requires sustained BMP signaling in these regions (Fig. 7; Onichtchouk et al. 1999). BAMBI is closely related to the product of a human gene, *nma*, that was identified by its low expression in metastatic melanoma cell lines compared to non-metastatic melanoma lines (Degen et al. 1996). The role of BAMBI/Nma in the adult and its involvement in the suppression of melanoma metastasis remain to be determined.

The EGF–CFC family: coadjuvants of Nodal signaling

In a class of their own, and acting through a mechanism that remains to be elucidated at the biochemical level, a group of secretory proteins that include Cripto and Cryptic in the mouse, and OEP in zebrafish, function as critical cofactors of Nodal signaling during various steps in the establishment of the body plan (Strahle et al. 1997; Ding et al. 1998; Schier and Talbot 1998; Gritsman et al. 1999; Saijoh et al. 2000). These proteins contain a motif with the predicted three-dimensional structure of epidermal growth factor (EGF), but unlike growth factors of the EGF family, the EGF–CFCs may not signal through receptor tyrosine kinases. Instead, it has been suggested that these proteins act in a cell-autonomous fashion, as membrane-tethered components that directly or indirectly support the signaling function of the Nodal receptor complex (for review, see Schier and Shen 2000).

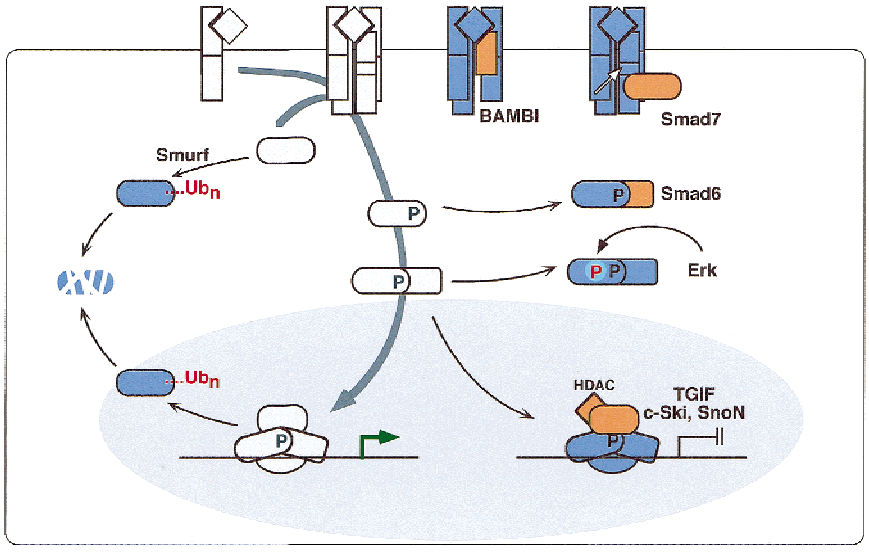

Figure 6. Smad pathway inhibitors. The basic pathway (gray), the inhibitors (orange) and the inhibited states (blue) are shown. BAMBI is structurally related to type I receptors and acts as a decoy that inhibits receptor activation. Smad7 acts as a R-Smad decoy that competes for the activated receptor kinase. Smad6 acts as a co-Smad decoy that competes for BMP-activated R-Smads. Smurf-1 mediates ubiquitin-dependent degradation of BMP-responsive Smads. A distinct ubiquitin-dependent degradation process specifically clears activated Smads from the nucleus. Erk-mediated phosphorylation in the linker region attenuates Smad nuclear accumulation. In the nucleus the transcriptional activity of a Smad complex can be negatively regulated by corepressors such as TGIF, c-Ski, and SnoN.

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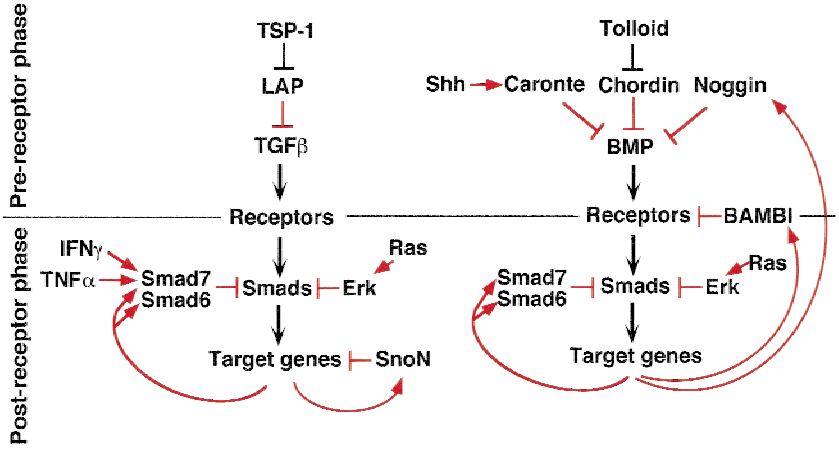

Figure 7. Pre- and postreceptor phases of the TGF-β and BMP signaling pathways: Feedback and integration with other pathways. Only a few examples of the regulators described throughout the text are shown to illustrate that the two phases of a TGF-β pathway can be similarly elaborate (black arrows) and highly regulated by feedback loops and inputs from other signaling pathways (red arrows).

Other receptor interactions

Several receptor-interacting proteins have been identified by yeast two-hybrid screenings. Little is known about the function of these proteins, but they could be involved in receptor regulation or signal propagation. Three of these proteins—TRIP-1 (TGF-β-receptor interacting protein-1), STRAP (serine-threonine kinase receptor-associated protein), and a regulatory subunit of protein phosphatase 2A (PP2A)—have in common the presence of WD protein–protein interaction domains. TRIP-1 associates with, and is phosphorylated by, TβR-II in a TGF-β-independent manner (Chen et al. 1995). TRIP-1 overexpression can inhibit a Smad-dependent transcriptional response but does not inhibit Smad activation (Choy and Derynck 1998). TRIP-1 is a component of translation initiation factor complex eIF3, raising the possibility that the TGF-β receptor may control the activity of this complex (Asano et al. 1997).

STRAP and the B-α subunit of PP2A have been identified as TβR-I-interacting proteins. When overexpressed, STRAP can associate with both TβR-I and TβR-II in a ligand-independent manner and impair TGF-β signaling, perhaps by recruiting the inhibitor Smad7 (see below) (Datta et al. 1998). The PP2A B-α subunit can associate with, and be phoshorylated by, TβR-I, suggesting that TGF-β may regulate the activity of PP2A (Griswold-Prenner et al. 1998).

TRAP-1 (TβR-I-associated protein-1) specifically recognizes the activated TβR-I (Charng et al. 1998). Overexpression of a TRAP-1 carboxy-terminal fragment attenuates TGF-β signaling. DIAP-1 and DIAP-2 (*Drosophila* inhibitor of apoptosis) bind to the *Drosophila* Dpp type I receptor, Thick veins (TKV) in a kinase-independent manner (Oeda et al. 1998). The role of these interactions in the regulation or mediation of TGF-β signaling remains largely unknown.

Controlling Smad access to the receptors

To function as intracellular mediators for TGF-β signals, the Smads must gain access to the receptors, undergo phosphorylation, form activated complexes, and accumulate in the nucleus. Not surprisingly, each of these steps appear to be tightly controlled.

Anchoring Smads for receptor activation

SARA (Smad anchor for receptor activation) is a Smad-interacting protein that facilitates the access of R-Smads to activated TGF-β receptors (Tsukazaki et al. 1998). It is a large protein with a central FYVE domain and a contiguous domain that binds Smad2 and Smad3 but not Smad1. The FYVE domain is a zinc finger-like structure that, in other proteins, has been shown to bind phosphatidylinositol-3-phosphate on the cytoplasmic surface of endosomal vesicles (Wurmser et al. 1999). Overexpression of SARA causes the clustering of Smad2/3 into a punctate pattern consistent with an association with endosomal vesicles. The carboxy-terminal domain of SARA may bind to the activated TGF-β receptor complex, bridging the receptor and Smad2/3. SARA overexpression increases the efficiency of receptor-mediated Smad2/3 phosphorylation. The resulting phosphorylation causes the release of Smad2/3 from SARA, allowing Smad movement to the nucleus (Tsukazaki et al. 1998).

SARA binds to the MH2 domain of Smad2 in an extended conformation, making serial hydrophobic contacts over the surface of the MH2 domain, but allowing full exposure of the putative receptor-interacting regions of Smad2 (Wu et al. 2000). Mutation of a Smad2 asparagine residue that is critical for the interaction with SARA (and is conserved in Smad3) prevents this interaction and decreases Smad2-dependent signaling (Wu et al. 2000). This residue is replaced by a serine in all BMP-regulated Smads, where it may be critical for interactions with SARA-like molecules in BMP pathways.

FYVE domains are present in diverse proteins involved in endocytic vesicular traffic (Wurmser et al. 1999). The possible association of SARA with endocytic vesicles raises interesting questions about the dynamics that the TGF-β receptor complex and the SARA–Smad2 complex must undergo to make their encounter. The receptor complex formed at the plasma membrane may have to be internalized to reach the SARA-bound Smads, and SARA

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itself might assist in this process by capturing the internalized receptor complex via the SARA carboxy-terminal region.

The fact that overexpression of SARA can alter the subcellular distribution of endogenous Smad2/3 (Tsukazaki et al. 1998) suggests that the endogenous level of SARA can bind only a fraction of the endogenous Smad2/3. It has been claimed that a large portion of the Smad2/3 pool in the cytoplasm is bound to the microtubule cytoskeleton, and this interaction is terminated by the action of activated TGF-β receptors (Dong et al. 2000).

Control of Smad levels by the ubiquitin ligase Smurf-1

Smurf-1 (Smad ubiquitination regulatory factor-1) is an E3 ubiquitin ligase identified as a Smad1-interacting protein (Zhu et al. 1999). Smurf-1 contains a HECT domain, which is typical of a group of E3 enzymes, and a WW protein–protein interaction domain that recognizes the PY motif (a proline-rich sequence with a tyrosine; PPXY in Smads). R-Smads contain a PY motif in the linker region (Fig. 3). However, the WW domain of Smurf-1 selectively recognizes Smad1 and Smad5, not Smad2 or Smad3 (or Smad4). The interaction with Smurf-1 leads to ubiquitination and degradation, effectively decreasing the steady-state levels of Smad1 and Smad5. In *Xenopus*, the expression pattern of *Smurf-1* overlaps that of *Smad1*. Overexpression of ectopic Smurf-1 in *Xenopus* embryos inhibits BMP/Smad1 signals, as evidenced by the dorsalization of ventral mesoderm and ectoderm neuralization. Smad1 binding and degradation by Smurf-1 occurs independently of activation by BMP signals. Thus, it appears that the primary function of Smurf-1 may be to adjust the basal level of Smads available for signaling by BMP pathways (Fig. 6). The signals that regulate Smurf-1 activity and the circumstances under which Smurf-1 exerts its effects remain unknown.

Antagonistic Smads in feedback and crosstalk

In addition to R-Smads and co-Smads, which carry signals from receptors to the nucleus, a third group of Smads act antagonistically, abrogating TGF-β signal transduction. The antagonistic Smads include Smad6 and Smad7 in vertebrates, Dad in *Drosophila*, and possibly Daf-3 in *Caenorhabditis elegans*. They contain a carboxy-terminal MH2 domain but have very little similarity to a cannonical MH1 domain in the amino-terminal region. The antagonistic Smads are known to mediate negative feedback within TGF-β signaling pathways and regulatory inputs from other pathways.

Smad7 inhibits Smad phosphorylation by occupying type I receptors for TGF-β, Activin, and BMP (for review, see Heldin et al. 1997; Massagué 1998) (Fig. 6). Mouse Smad7 preferentially inhibits Activin and TGF-β signaling over BMP signaling (Souchelnytskyi et al. 1998; Ishisaki et al. 1999). The reverse is true of a *Xenopus* Smad7 homolog (Souchelnytskyi et al. 1998). Smad7 appears to reside predominantly in the nucleus at basal state and translocates to the cytoplasm upon TGF-β stimulation (Itoh et al. 1998). The significance of this phenomenon remains to be elucidated.

Smad6 preferentially inhibits BMP signaling by a mechanism different from that of Smad7 (Hata et al. 1998; Ishisaki et al. 1999). When expressed at levels that are sufficient for inhibition of BMP signaling but not TGF-β signaling, Smad6 does not interfere with receptor function but competes with Smad4 for binding to receptor-activated Smad1 and yields inactive Smad1–Smad6 complexes (Fig. 6). Overexpression of Smad4 can outcompete Smad6 and rescue BMP signaling (Hata et al. 1998). At higher expression levels, Smad6 can mimic Smad7 and inhibit signaling by BMP and TGF-β receptors (Imamura et al. 1997). *Smad6*-defective mice have multiple defects in the development and homeostasis of the cardiovascular system (Galvin et al. 2000). The ossification of the aorta in these animals, in particular, is suggestive of an excess of BMP signaling activity. *Drosophila* Dad antagonizes Dpp signaling in the control of anteroposterior patterning of the wing imaginal disc (Tsuneizumi et al. 1997).

The expression of both Smad6 and Smad7 is increased in response to BMP, Activin and TGF-β, suggesting roles in negative feedback of these pathways (Nakao et al. 1997; Ishisaki et al. 1998, 1999) (Fig. 7). *Smad6* expression in the developing chick heart can be diminished by ectopic Noggin and augmented by ectopic BMP2, suggesting that a BMP negative feedback loop via Smad6 has a role in orchestrating BMP-mediated cardiac development (Yamada et al. 1999). Similarly, Dpp induces the expression of its own antagonist Dad in *Drosophila* (Tsuneizumi et al. 1997).

The expression of Smad7 can also be increased by pathways that negatively regulate TGF-β signaling (Fig. 7). One example is provided by the ability of interferon-γ (IFN-γ), acting via the Jak1 tyrosine kinase and the Stat1 transcription factor, to increase Smad7 expression (Ulloa et al. 1999). As a result, IFN-γ inhibits TGF-β-mediated Smad3 phosphorylation and signal transduction. Thus, Smad7 induction by IFN-γ provides a mechanism for transmodulation between the STAT and SMAD signal-transduction pathways, providing a basis for the known antagonism between TGF-β and IFN-γ in the regulation of immune cell functions. A similar set of events has been shown to occur in response to the proinflammatory cytokines tumor necrosis factor-α and interleukin-1β, which activate *Smad7* expression via the NF-κB/RelA transcription factor (Bitzer et al. 2000).

Regulation of Smad accumulation in the nucleus

Little is known about the mechanisms mediating the nuclear accumulation of Smads in response to TGF-β family agonists. However, the nucleus accumulation of Smads is known to be the target of regulatory inputs from other signaling pathways, in particular the Ras pathway, and is limited by the presence of a ubiquitin-

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dependent degradation process that specifically targets Smads in the nucleus.

Inhibition of Smad nuclear accumulation by Ras-activated Erk kinases

In epithelial cells, TGF-β acts as a cytostatic agent by dominating over the mitogenic effect of Ras-activating growth factors, whereas transformation of epithelial cells with oncogenically activated *ras* alleles overrides the antiproliferative effect or TGF-β (Longstreet et al. 1992; Oft et al. 1996; Calonge and Massagué 1999; Kretzschmar et al. 1999). Additional examples of antagonism between Ras-activating factors and TGF-β family members are provided by the opposite roles of FGF and BMP signaling in limb and tooth morphogenesis (Niswander and Martin 1993; Ganan et al. 1996; Neubuser et al. 1997).

Ras signaling can directly interfere with Smad-dependent responses by attenuating the agonist-induced nuclear accumulation of Smad1, Smad2, and Smad3 (Kretzschmar et al. 1997, 1999). This effect is mediated by the phosphorylation of Smad1, Smad2, and Smad3 by Ras-activated Erk1 and Erk2 protein kinases. Clusters of four consensus Erk sites (PxS/TP sequence) in the linker region of Smad1, or one Erk site and three SP sites in Smad2 and Smad3 (see Fig. 3), are the targets of Erk-mediated phosphorylation. Phosphorylation of these sites can be induced by cell stimulation with EGF or HGF or by expression of oncogenic Ras. Alanine mutation of these sites renders the Smads resistant to these inhibitory effects (Kretzschmar et al. 1997, 1999). This Smad3 mutant partially restores TGF-β antiproliferative responses in Ras-transformed epithelial cells. Smads therefore receive opposing regulatory inputs from Erk kinases and TGF-β family receptors, providing a basis for the counterbalanced regulation of Smads by these two types of pathways.

Smad proteins have been shown to interact with the calcium-binding protein calmodulin in vitro and in transfected cells (Zimmerman et al. 1998). Overexpression of calmodulin inhibits the response of a TGF-β transcriptional reporter, but the mechanism of this effect is unknown. It is noteworthy that in addition to Erk phosphorylation sites and a PY motif, the liker region of Smad2 and Smad3 contains consensus sites for calcium/calmodulin-dependent protein kinase (Fig. 3; Feinmesser et al. 1999).

Smad clearance from the nucleus by ubiquitin-dependent degradation

Accumulation of receptor-phosphorylated Smad2 is a transient process. The decline in phosphorylated Smad2 that eventually occurs after stimulation with TGF-β appears to be largely due to proteasome-mediated degradation (Lo and Massagué 1999). The pool of activated Smad2 in a TGF-β-treated cell is subject to constant culling by the ubiquitin/proteasome pathway. In the presence of a proteasome inhibitor, degradation of phosphorylated Smad2 is averted, and Smad2 stays active in the nucleus. The generation of multiubiquitinated Smad2 in response to TGF-β requires receptor-mediated phosphorylation of the carboxy-terminal serines. However, this phosphorylation seems to be necessary for Smad2 ubiquitination to the extent that it is required for nuclear accumulation of Smad2. Smad constructs that constitutively accumulate in the nucleus by being tagged with a nuclear localization signal undergo constitutive ubiquitination. Proteasome degradation of Smad2 does not appear to require export of Smad2 from the nucleus.

Ubiquitin conjugation to protein substrates requires ubiquitin-conjugating enzymes, also known as E2 enzymes, which transfer activated ubiquitin to the substrate, either directly or via E3 ubiquitin ligases. The E2 UbcH5 has been implicated in the ubiquitination of nuclear Smad2 (Lo and Massagué 1999). The E3 enzyme involved in this process is not known but is unlikely to be a WW domain protein like Smurf-1 because Smad2 constructs lacking the linker region (thus lacking the PY motif for WW domain recognition) still undergo ubiquitination in the nucleus. The exact role of ubiquitin-dependent degradation of Smad2 remains to be defined. TGF-β and related factors regulate extremely dynamic physiological processes. Ubiquitin-dependent degradation of their activated mediators may ensure a swift elimination of their signal. Alternatively, ubiquitination might selectively remove the surplus of activated Smad from the nucleus by targeting Smad that is not bound to target promoters or to other partners.

Smad transcriptional corepressors as effectors and regulators

An incoming Smad complex in the nucleus may associate with transcriptional coactivators or alternatively with transcriptional corepressors (for review, see Massagué and Wotton 2000). Three Smad corepressors recently have been identified: the homeodomain protein TGIF (Wotton et al. 1999), and the two related proteins c-Ski and SnoN (Akiyoshi et al. 1999; Luo et al. 1999; Sun et al. 1999a). All three interact with the MH2 domain of Smad2 and Smad3. TGIF has been shown to interact directly with histone deacetylases (HDACs), and recruit HDACs to an endogenous Smad2–Smad4 complex in response to TGF-β (Wotton et al. 1999). Ski and SnoN interact with NCoR, which can recruit HDACs (Luo et al. 1999).

TGF-β and related factors are known to have gene inhibitory responses. The TGF-β-induced association of Smads with TGIF may underlie some of these responses. Additionally, however, interaction with corepressors could serve a regulatory purpose either in the suppression of Smads that may leak into the nucleus in the basal state, or in imposing limits to positive gene responses induced by agonist-activated Smads. Evidence for both of these roles has been provided. Ski and SnoN are found associated with Smad3 in the basal state (Luo et al.

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1999). Upon TGF-β stimulation, these repressors may undergo rapid degradation, their levels increasing later and possibly contributing to turn off Smad-dependent gene activation (Stroschein et al. 1999; Sun et al. 1999b). Cell treatment with *TGIF* antisense oligonucleotides increase transcriptional responses to TGF-β, providing evidence that endogenous TGIF may place a limit on the amplitude of these responses (Wotton et al. 1999).

Additional inhibitors of the nuclear functions of Smads include SIP1 (Smad-interacting protein 1) and Evi-1. SIP1 interacts with the MH2 domain of Smad1, Smad2, Smad3, and Smad5, but not with Smad4, in both yeast and mammalian cells (Verschueren et al. 1999). SIP1 is a zinc finger/homeodomain protein and a transcriptional repressor that can bind to the *Xenopus Xbra2* promoter and repress *Xbra* expression. Evi-1 is a zinc finger protein that stimulates cell growth, inhibits differentiation, and promotes myeloid leukemia formation. Evi-1 interacts with Smad3, but not with other Smads, and is able to inhibit the activation of TGF-β-responsive reporters and attenuate TGF-β-induced growth inhibition (Kurokawa et al. 1998). Evi-1 does not interfere with Smad3 activation but prevents binding of the Smad3–Smad4 complex to DNA. The roles of endogenous SIP1 and Evi-1 in TGF-β or BMP signaling remain to be determined.

Integrating a signaling network

The negative regulation of Smad accumulation in the nucleus by Erk-mediated phosphorylation of the linker region of R-Smads is but one among a growing number of examples of how the TGF-β family signaling pathways operate as part of a signaling network that collects and integrates diverse environmental cues in the cell.

Dual effect of Ras signaling on Smad-dependent responses

An extensive body of evidence indicates that the Ras and Smad pathways can interact at different levels and with different outcomes, depending on the cellular context. Responses that are directly proportional to the level of Smad activity in the nucleus may be attenuated by the opposing effects of Ras signaling, as is the case with the antiproliferative response to TGF-β in epithelial cells (Longstreet et al. 1992; Oft et al. 1996; Calonge and Massagué 1999; Kretzschmar et al. 1999). Antagonism between the TGF-β and Ras pathways can occur at multiple levels. For example, in the control of epithelial cell proliferation, the opposite effects of Ras activators and TGF-β at the level of Smad nuclear accumulation are part of a response that also includes opposite effects on cyclin-dependent kinases (Cdks) during the G_1 phase of the division cycle: Ras signaling stimulates the activation of Cdks, whereas TGF-β signaling induces expression of various Cdk inhibitors that cancel the effects of Cdk activation by Ras (Hannon and Beach 1994; Reynisdóttir et al. 1995).

The interaction between TGF-β and Ras signaling can also be cooperative, resulting in outcomes that neither pathway would achieve on its own. Oncogenic Ras in mammary epithelial cells not only attenuates Smad-mediated antiproliferative responses but also endows these cells with the ability to respond to TGF-β with transdifferentiation into a highly invasive and metastatic phenotype (Oft et al. 1996, 1998). Breast cancer cells with a hyperactive Ras pathway (owing to EGF receptor gene amplification) respond to TGF-β with an increased ability to metastasize to bone (Yin et al. 1999). Thus, oncogenic Ras does not merely block Smad signaling, but it "reprograms" the TGF-β response of epithelial cells.

Cooperative effects between Ras and TGF-β family signals are also observed in embryonic development. Ras signaling cooperates with Activin-like signaling during mesoderm induction in *Xenopus* (Whitman 1998). Dpp signaling and signaling by receptor tyrosine kinases are interdependent in the determination of cell fates in the *Drosophila* embryonic mesoderm (Carmena et al. 1998) and in endoderm induction (Szuts et al. 1998). Responses that may depend on a certain level of Smad activity in the nucleus, such as those characteristic of morphogen gradients, may require the counterbalancing effect of Ras signaling to achieve a suitable level of nuclear Smad activity. Cooperation between these pathways can also occur as a result of their concommitant, but otherwise independent inputs into common target promoters. For example, in the induction of endoderm, the transcriptional response elements for the Dpp signal in midgut enhancers from homeotic target genes are bipartite, comprising Ras-responsive CRE sites as well as binding sites for Dpp-activated Smads (Szuts et al. 1998).

Synergies with JNK and p38 kinase pathways

A growing body of work provides evidence that TGF-β and BMP can activate various MAPK signaling pathways, most prominently the MKK4–JNK and MMK3–p38 pathways (Afti et al. 1997; Adachi-Yamada et al. 1999; Hocevar et al. 1999; Iwasaki et al. 1999; Sano et al. 1999) These responses and their kinetics are various, depending on the cell type (Hocevar et al. 1999; Iwasaki et al. 1999). The biochemical link between the receptors and these pathways is not clear but may involve the protein kinase TAK1 (TGFβ-activated kinase 1) acting directly on MKK enzymes that activate either JNK or p38 (Shibuya et al. 1996; Zhou et al. 1999). It has been suggested that a direct link between TAK1 (in association with the cofactor TAK-binding protein 1, TAB1) and the receptors may be established by yet another upstream kinase, HPK1, in the case of TGF-β (Zhou et al. 1999), or via a physical association with the protein designated IAP (inhibitor of apoptosis) in the case of BMP (Yamaguchi et al. 1999). TAK-1 appears to be a multifuntional mediator also involved in the interleukin-1 signaling pathway and as a negative regulator of the β-catenin/TCF pathway (Ishitani et al. 1999; Ninomiya-Tsuji et al. 1999).

Regardless of the receptor-coupling mechanism in-

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volved, TGF-β activation of JNK or p38 in some cell lines and conditions can be rapid and mediate transcriptional responses by activating AP-1 complexes via phosphorylation of c-Jun transcription factor (Hocevar et al. 1999) or CRE-regulatory complexes via phosphorylation of ATF2 transcription factor (Sano et al. 1999). This may result in the generation of separate signals that converge with Smads in the activation common target promoters. Taking this possibility one step further, it has been reported that activated Smad complexes can form physical interactions with Jun complexes (Zhang et al. 1998; Wong et al. 1999) or ATF-2 complexes (Hanafusa et al. 1999; Sano et al. 1999). Furthermore, Smads themselves have been reported to undergo activating phosphorylation by JNK on as yet unidentified sites in the linker region (J.D. Brown et al. 1999; Engel et al. 1999). The interplay between the Smad and JNK or p38 pathways could underlie diverse forms of integration and reciprocal regulation between TGF-β signaling and other pathways in the cell.

Smad links with other pathways

In the *Xenopus* embryo, the Wnt and Smad2 signaling pathways may reciprocally cooperate to induce the expression of SO specific genes (Crease et al. 1998). Smad2 signaling can enhance the ability of Wnt, acting via β-catenin, to induce *siamois*, whereas the Wnt pathway can enhance induction, by the Smad2 pathway, of the organizer genes *goosecoid* and *chordin*. Smad4 cooperates with β-catenin in the activation of twin (Nishita et al. 2000).

OAZ, the Smad1 DNA-binding cofactor that mediates activation of the ventral mesoderm homeotic gene *Vent.2* in the BMP pathway, is a protein with 30 zinc fingers that associates with a BMP-induced Smad1–Smad4 complex and binds the BMP-response element of the *Xvent.2* promoter (Hata et al. 2000). The sets of zinc fingers that are not devoted to these interactions function in a separate pathway as mediators of interactions with Olf/EBF-1, a transcription factor implicated in the development of the olfactory epithelium and pre-B lymphocytes in mammals. These two functions of OAZ appear to be mutually exclusive, providing an additional device for the integration of diverse pathways, in this case at the level of a Smad transcriptional partner.

Conclusions

TGF-β, Activin, and BMPs are multifunctional cytokines, but their signal transduction pathways are based on a relatively simple central signaling engine. The progressive elucidation of the elaborate mechanisms that control this system is shedding light on the general principles that govern TGF-β signaling and its integration with regulatory networks of the cell.

It is now apparent that TGF-β signaling pathways have equally important extracellular and intracellular—or prereceptor and postreceptor—phases (Fig. 7). Outside the cell, the processes of agonist sequestration, activation, and controlled diffusion establish prereceptor pathways of signal relay that can be as elaborate and biologically important as the postreceptor events that convey the signal to the nucleus. Both the pre- and postreceptor phases of a TGF-β pathway can have similar types of regulators. For example, inhibitors such as Noggin and BAMBI may constrain the initial step—activation of the factor or the receptor, respectively—in each of these phases in a BMP signaling pathway. Accessory factors can enhance the binding of an agonist to its cognate receptor (e.g., Betaglycan-enhancing ligand binding to TGF-β receptors) and the binding of a receptor-activated Smad to its target promoter (e.g., FAST enhancing the binding of Smad2 to *Mix.2*). Furthermore, both in the prereceptor and postreceptor phases, regulators can mediate feedback control, as Noggin and BAMBI do, respectively in the BMP pathway, or regulatory inputs from other pathways (e.g., Caronte-inhibiting BMPs in response to Shh- and Smad7-inhibiting Smad3 activation in response to IFN-γ).

It is also apparent that the simplicity of the Smad signaling engine can give rise, at the transcriptional level, to highly complex patterns of gene expression. This is most apparent during embryo development, in which the Smad1 and Smad2 pathways lead to the activation of many homeotic genes; these, in turn deploy extensive programs of gene expression. One of the major tasks ahead will be to further delineate the roles and specificity of the components that direct TGF-β signaling pathways to concrete targets in normal physiology and to aberrant targets in the altered conditions of disease states.

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