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INSIGHTS FROM MODEL SYSTEMS
Specificity in Transforming Growth Factor–β Signaling Pathways

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We have recently seen an explosion of information in the transforming growth factor (TGF)–β field, and it is remarkable to consider that the receptors and signal transducers for the TGF-β superfamily were identified only over the past several years. With at least 30 TGF-β superfamily members, the diversity of biological processes influenced by these cytokines is not a surprise; however, we are now beginning to appreciate the complexity of signaling, as the multiple layers of regulation are revealed.

Members of the TGF-β superfamily of cytokines influence a diverse range of normal cellular processes, such as the secretion of the extracellular matrix, cell adhesion, cell proliferation, and apoptosis, and therefore are well placed to effect the morphogenesis of tissues and organs and even the overall body plan of a developing embryo. In addition, alteration of TGF-β signaling has been linked to various developmental abnormalities, cancer progression, and other human disorders. The variety of effects that have been attributed to TGF-β superfamily members underscores the importance of TGF-β signaling and highlights the question of how this ligand family exerts specific effects, depending on the cellular context.

The ability of TGF-β superfamily members to direct tissue morphogenesis is illustrated by the activities associated with the bone morphogenetic proteins (BMPs), one major group within the superfamily (Hogan 1996). BMPs were originally identified as molecules that could induce ectopic bone formation and, as the name suggests, are thought to affect bone morphogenesis. The BMPs are heterogeneously expressed during skeletal development, and, in both the mouse and human, mutations in BMPs are associated with various chondrodysplasias (Hogan 1996; Massague 1998). In addition to bone morphogenesis, TGF-β signaling appears to play a role in vascular histogenesis. Mutations in either of two genes encoding receptors of the TGF-β pathway have been linked to the autosomal dominant disorder hereditary hemorrhagic telangectasia (HHT), which presents with vascular malformations in the skin and mucosa (McAllister et al. 1994; Johnson et al. 1996). This link, together with the demonstration that these receptors are predominantly expressed in vascular endothelial cells, suggests that they are important in the regulation of the TGF-β response during the development and homeostasis of the vasculature.

Evidence from vertebrate and invertebrate systems suggests that TGF-β signaling also plays a more global role in the morphogenesis of the entire embryo. Here, we focus primarily on experiments performed by use of the frog *Xenopus laevis,* where classic models of pattern formation during embryogenesis are being tested with modern molecular tools (see sidebar). In *Xenopus,* as in the mouse and the fruit fly *Drosophila melanogaster,* TGF-β signaling, specifically that of the BMP ligands, is thought to play an important role in the specification of the dorsoventral axis of the embryo (DeRobertis and Sasai 1996; Hogan 1996; Lawrence and Struhl 1996; Harland and Gerhart 1997). The involvement of BMPs in both bone morphogenesis and embryonic patterning illustrates the ability of TGF-β ligands to elicit differential responses, depending on the cellular context. Thus, how signaling specificity is achieved is a central question in the TGF-β field. At the receptor level and both intra- and extracellularly, this pathway offers diversity and combination opportunities that are likely to underlie the multifunctional nature of the TGF-β superfamily. Thus, the purpose of this review is twofold: by use of *Xenopus* as a model system, (1) to introduce the reader to molecular embryogenesis and (2) to suggest mechanisms whereby signaling specificity can be achieved.

The TGF-β Signaling Pathway

The currently accepted model of the TGF-β signaling pathway is shown in somewhat simplified form in figure 1. According to this model, signaling begins when a specific ligand binds to its cognate type II receptor, a serine/threonine kinase, which then heterodimerizes with and phosphorylates a distinct serine/threonine kinase, the

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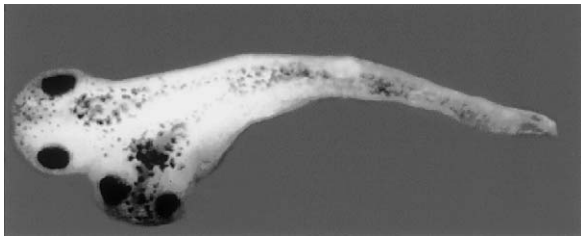
Getting Organized

Classic embryological experiments using grafting techniques defined a region of the embryo, known as the "organizer," that had the ability to induce and pattern competent tissue in a host embryo, when transplanted ectopically. The search soon ensued for molecules that could mimic the organizer's effects. Modern molecular embryologists have used the introduction of ectopic genes into *Xenopus* embryos, by cytoplasmic RNA or DNA injection, to identify the molecules involved in the organizer's activity. *Xenopus* oocytes or embryos can be injected at various stages, depending on the needs of the particular experiment. A molecule is said to have "organizer activity" when, after injection into the ventral side of the embryo, it can induce ectopic neural and mesodermal tissues. In particularly dramatic cases, ectopic tissues may even form an entire duplicated embryo, also known as a "complete secondary axis." In the example below, embryos were injected ventrally at the four-cell stage with BVg1, a TGF-β superfamily member, and a complete secondary axis formed that included both head and trunk.



Dorsal injections can result in various phenotypes, ranging from hyperdorsalization to complete ventralization, and are rated by an established dorsoanterior index. Various permutations of these standard injections can be performed. For example, "secondary axis rescue" occurs when a molecule known to induce secondary axes is coinjected, on the ventral side of the embryo, with one thought to counteract its effects. If the secondary axis is reduced or abolished, the second molecule is said to "rescue" the secondary axis.

type I receptor. This activated type I receptor, in turn, phosphorylates one of several pathway-specific smad proteins. The smads are intracellular signal transducers of the TGF-β pathway, and they can be roughly categorized as (1) pathway-specific smads, including smads 1–3, 5, and 8, which are directly phosphorylated by type I receptors; (2) common or shared smads (of which smad4 is the only known member), which act in multiple TGF-β–superfamily signaling pathways; (3) inhibitory smads, such as smad6 and smad7, which block the transduction of TGF-β signals. Once phosphorylated by its cognate type I receptor, the pathway-specific smad becomes activated, permitting heterodimerization with a common smad; the complex is then translocated to the nucleus, where it effects transcription (Massague 1998; Whitman 1998). The smads appear to bind DNA through a conserved region known as the "MH1 domain," and they activate transcription via a region known as the "MH2 domain." The smads also assemble with nuclear transcription factors to affect target-gene expression.

This model implies that specificity of TGF-β signaling is dictated by the type I receptor and, by extension, the particular pathway-specific smad that it activates. However, further modulation of signaling may occur within the cell, via smad interaction with other factors and cross talk with other signaling components (Chen et al. 1996; Kretzschmar et al. 1997). In addition, there is evidence that extracellular components, as well as accessory receptors, are important in the modulation of the TGF-β response, thus providing another level of regulation (McAllister et al. 1994; DeRobertis and Sasai 1996; Marques et al. 1997; Piccolo et al. 1997). Although this model provides the beginnings of a molecular framework of how TGF-β signaling occurs, our understanding of how TGF-β superfamily members regulate cell-type–specific transcriptional activation is incomplete.

Ligand Regulation of Response and the Morphogen Hypothesis

During early *Xenopus* embryogenesis, the dorsal and ventral domains of the mesodermal germ layer are

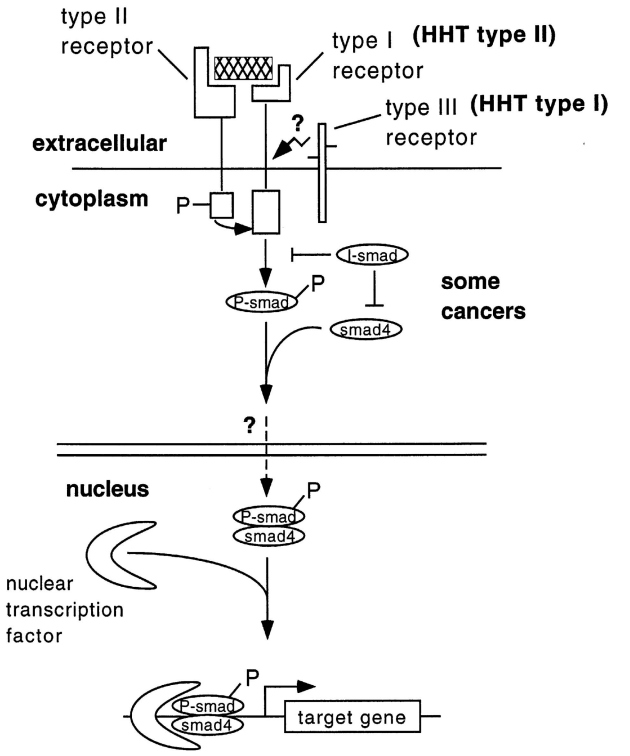


Figure 1 Schematic of the components of the TGF-β signal-transduction pathway. Disorders associated with various components of the pathway are indicated. P-smad = pathway-specific smad, and I-smad = inhibitory smad.

thought to be determined by activin/BVg1 and BMP signals, respectively, both of which are members of the TGF-β superfamily of cytokines (Harland and Gerhart 1997). However, if activin/BVg1 signals are responsible for specifying the dorsal mesodermal domain, how are the various dorsal cell types (such as notochord and muscle cells) generated in response to a single ligand? The answer may lie in the ability of TGF-β superfamily members to act as morphogens—that is, as factors that elicit differential, patterned responses within a tissue, in accordance with the amount of ligand presented to the cells within the tissue (Gurdon et al. 1994; Harland and Gerhart 1997). For example, graded amounts of activin have been shown to specify various mesodermal cell types, with higher levels specifying the dorsal-most types and lower levels specifying the more ventral types. At the molecular level, high levels of activin can induce the dorsal mesodermal marker *goosecoid* (*gsc*), and lower levels of activin can induce the mesodermal marker *brachyury* (*Xbra*). Similarly, BMP4, the vertebrate homologue of *Drosophila decapentaplegic* (*dpp*), also has recently been shown to behave as a morphogen in *Xenopus* embryos (Dosch et al. 1997).

How Do Different Ligand Concentrations Dictate Multiple Cell Fates in Responding Tissues?

Recruitment of heterogeneous type I and type II receptor combinations with distinct signaling capacities by variation of concentrations of ligand, perhaps due to varying dissociation constants (K_d) among the receptors, provides a means by which variation could be achieved by a single signaling molecule. For instance, activin can form a complex with activin receptor (ActR)–IIB and either the ActR-I or ActR-IB receptor, and each combination yields a unique physiological outcome (Carcamo et al. 1994; Armes and Smith 1997). However, other mechanisms may account for these effects, since differential responses also can be elicited by varying concentrations of a single type I receptor (Armes and Smith 1997). Consistent with these data, variations in type I–receptor concentration also can elicit patterned responses in the *Drosophila* wing (Lecuit and Cohen 1998). In this case, a feedback loop involving *dpp* negatively regulates the amount of receptor expression, which, in turn, influences the effective range of the dpp gradient.

Cells may also detect ligand concentration by reading the total number of occupied receptors per cell, as has been elegantly shown by Dyson and Gurdon (1998) for the case of the ActR-IIB receptor in *Xenopus* blastomeres. These authors demonstrated that cells induce *Xbra* expression at low levels of ActR-IIB occupancy and *gsc* expression at high levels, rather than by reading out a ratio of occupied to unoccupied receptors. Because signaling specificity is thought to occur via type I receptors, it is unclear how type II–receptor occupancy is interpreted by the type I/II–receptor complex. However, similar responses to increasing activin doses were elicited in tissues overexpressing either ActR-I or ActR-IB mRNA, leading Dyson and Gurdon to conclude that, although signaling specificity is mediated by type I receptors, the identity of the type I receptor does not qualitatively influence the response to high or low ligand concentrations. These data demonstrate that activin exhibits properties of a morphogen in experimental situations, but it remains unclear whether activin functions as a morphogen in vivo or, indeed, whether a gradient of activin protein actually exists in the embryo. Using a luciferase-reporter construct containing an activin-responsive element, Watabe et al. (1995) demonstrated that activin activity appeared to be equivalent in domains of endogenous *gsc* and *Xbra* expression, implying that levels of activin (or an activin-like molecule) are equivalent throughout the embryo. These results suggest that a gradient of activin concentration across the tissue may not be solely responsible for the differential activation of these genes in vivo.

Binding of TGF-β ligands to their receptors can be modulated, in some cases, by non–signal-transducing accessory receptors. The transmembrane proteoglycan betaglycan and the related cell-surface glycoprotein endoglin belong to this third class of receptors, which is thought to indirectly effect TGF-β signaling by controlling access to the type I/II receptors (Massague 1998). Both endoglin and betaglycan bind TGF-β, although with lower affinity than the type II receptors, and both have been shown to associate with type I and type II receptors to form a multimeric receptor complex. The role of betaglycan as a facilitator of the binding of TGF-β to the signaling receptors is most evident with TGF-β2. This TGF-β isoform exhibits a low affinity for type I and type II receptors, but receptor binding and activity can be augmented in the presence of betaglycan (Massague 1998). Endoglin, in contrast, does not bind TGF-β2 but binds TGF-β1 and TGF-β3. Although its role in the binding of TGF-β to signaling receptors is unclear, endoglin has been demonstrated to have a functional role in endothelial cells, since it has been identified as the gene responsible for the autosomal dominant disorder HHT type I (McAllister et al. 1994). Interestingly, mutations in activin receptor–like kinase (ALK) 1, a type I cell-surface receptor for the TGF-β superfamily of ligands, are responsible for the similar disorder HHT type II (Johnson et al. 1996). The physiological ligand for ALK1 is still unknown, but the fact that mutations in both endoglin and ALK1 give rise to similar human disorders suggests that endoglin and ALK1 act in the same pathway and that endoglin may facilitate ligand binding to ALK1. A third possible member of this accessory-

receptor type is *dally,* a *Drosophila* member of the vertebrate glypican family of heparan sulfate–containing integral membrane proteoglycans. In *Drosophila, dally* has been linked genetically to *dpp* signaling, and mutations in human glypican 3 are responsible for Simpson-Golabi-Behmel syndrome in humans (Selleck 1999). Although a biochemical interaction between *dally*/glypican and *dpp* has not been described, these examples serve to illustrate the importance of accessory receptors in the regulation of TGF-β signaling.

Intracellular Mechanisms of TGF-β Regulation

Studies in *Xenopus* showed that overexpression of smad2 or its carboxy-terminal half can mimic the mesoderm-inducing effects of activin (Baker and Harland 1996; Graff et al. 1996), whereas overexpression of smad1 or smad5 mimics the ventralizing effects of BMP4 (Thomsen 1996; Suzuki et al. 1997). These observations suggest that different smads might transduce distinct TGF-β–superfamily signals. Further studies in *Xenopus,* with BMP2/4- and activin/BVg1-specific reporters, demonstrated that the specificity of the ventral BMP2/4 signals is mediated intracellularly by smad1 and that of the dorsal activin/BVg1 signals is mediated by smad2 (Candia et al. 1997). Although the response of a given cell to a particular TGF-β–superfamily signal is due in part to the activation of a particular pathway-specific smad in the cell, the question of how cells can respond differentially to the same ligand, by activation of a pathway-specific smad, still remains. Interestingly, ectopic expression of pathway-specific smads in *Xenopus* embryos can recapitulate, in some respects, the differential tissue inductions of varying concentrations of TGF-β–superfamily ligands. For example, ectopic expression of smad2 in prospective ectoderm is sufficient to induce relatively ventral mesodermal markers (e.g., *Xbra*) at low doses and dorsal markers (e.g., *gsc*) at high doses (Baker and Harland 1996; Graff et al. 1996), suggesting that the amount of smad2 activation by the receptor complex can regulate which target genes are activated. In these experiments, doses of smad2 that were high enough to induce *gsc* also induced *Xbra;* however, as noted by Artinger et al. (1997), doses of activin high enough to induce *gsc* inhibit expression of *Xbra,* which is induced by lower doses of activin. Thus, the effects of a gradient of ligand stimulation may be more complex than simple activation of a smad gradient.

Cellular responses to TGF-β superfamily members also involve negative regulation by inhibitory smads. The inhibitory smad6 and smad7 in vertebrates and *Dad* (*daughters against dpp*) in *Drosophila* participate in negative feedback loops that appear to regulate the intensity or duration of the TGF-β response (Imamura et al. 1997; Nakao et al. 1997; Tsuneizumi et al. 1997). The antagonism of the inhibitory smads appears to occur at the level of phosphorylation of the pathway-specific smads, with smad7 inhibiting TGF-β–mediated phosphorylation of smad2 and smad3 and with smad6 inhibiting the phosphorylation of smad1 and smad2 but not of smad3. This mechanism appears to involve the binding of the inhibitory smad to the type I receptor, thus preventing activation of the pathway by blocking the interaction of the pathway-specific smad with the receptor (Imamura et al. 1997; Nakao et al. 1997; Tsuneizumi et al. 1997). Evidence also suggests that, at low levels, smad6 inhibits smad1 by competing with smad4 for binding to activated smad1 (Hata et al. 1998). Thus, by attenuating the response to TGF-β ligands, inhibitory smads may be able to affect the gradient of TGF-β signals within a tissue.

In addition to their ability to act as transcriptional activators, smads can interact with other factors that together serve to modulate a cell's response to a particular ligand, thereby providing an additional level of regulation. In *Xenopus,* forkhead activin signal transducer (Fast)–1, a winged helix/forkhead transcription factor, and the related Fast-2 in mouse appear to be important in the induction of the early mesoderm response genes *mix.2* and *gsc,* respectively (Chen et al. 1996; Labbe et al. 1998). Both Fast-1 and Fast-2 can form a complex with smad2 and smad4, positively regulating an activin/TGF-β response element in the promoter of these target genes, in a ligand-dependent manner. Interestingly, smad3, a pathway-specific smad that is closely related to smad2, can block an activin/TGF-β–responsive *gsc*-reporter construct in a dose-dependent manner in cells transfected with Fast-2 and with smad2 and smad4 (Labbe et al. 1998). This inhibition appears to be mediated by the MH1 domain of smad3, possibly via its interaction with a smad4 binding site in the *gsc* promoter. Although the physiological relevance of such an interaction is still unclear, the differential effect of the related smad2 and smad3 suggests another mechanism whereby diverse responses to TGF-β ligands could be generated.

Extracellular Regulation of TGF-β Signaling

The classic experiments of Spemann and Mangold (1924) demonstrated that, on transplantation to the ventral side of the embryo, a region of dorsal mesoderm of the amphibian embryo that they termed the "organizer" was capable of inducing tissue normally specified as epidermis to become neural with the appropriate pattern. Furthermore, the organizer was capable of inducing more dorsal mesodermal cell types, such as muscle in more ventral tissue destined to become mesenchyme. Because the induction of an ectopic nervous system, as well as the dorsalization of ventral mesoderm, was dependent

on the presence of the organizer, ventral was assumed to represent a "default" state, and signals from the organizer were assumed to be required in order to induce and pattern neural and dorsal mesodermal tissues, presumably by positively activating specific signal-transduction pathways. Recent experiments, however, have challenged this dogma, suggesting that inhibitory signals from the organizer are required in order to antagonize the signals present in the ventral ectoderm and mesoderm rather than positively acting through their own receptors/signal transducers (see fig. 2). Our current understanding is that ventral signals mediated by BMP4 (but that are likely to involve BMP2 and BPM7 as well) induce epidermis and that neural fate results when this activity is relieved by extracellular antagonism from the organizer-secreted molecules noggin and chordin (Wilson and Hemmati-Brivanlou 1995; Piccolo et al. 1996; Zimmerman et al. 1996). Both noggin and chordin bind BMP ligands directly with high affinity, presumably preventing interaction with their receptors. Chordin is a homologue of the *Drosophila* gene *short gastrulation* (*sog*), which functions similarly in flies by sequestering *dpp* and *screw,* another *Drosophila* BMP homologue (DeRobertis and Sasai 1996; Nguyen et al. 1998). Noggin has similar activity when injected into *Drosophila* embryos, but an invertebrate homologue has not yet been identified. The presence of secretable BMP antagonists in embryos, at the appropriate place and time, suggests that they function in vivo to regulate the availability of BMP ligands, which ultimately results in the differential regulation of BMP target genes.

The ability of the organizer to induce and pattern ventral regions of the embryo may be related to the ability of TGF-β ligands to function as morphogens, which have the ability to elicit a differential pattern within a tissue in response to their concentration gradient. As was alluded to above, BMP4 recently has been shown to behave as a morphogen in dorsoventral mesoderm patterning in *Xenopus* (Dosch et al. 1997), and its homologue, *dpp* in *Drosophila,* previously had been proposed to act as a morphogen in the fly embryo, as well as in the wing imaginal disc (Lawrence and Struhl 1996). In the *Xenopus* model, BMP4 acts during dorsoventral mesoderm patterning to specify more ventral mesodermal cell fates with increasing concentrations. However, whether graded levels of BMP4 (or another BMP) protein exist in the embryo is not clear, since its message appears to be uniform throughout the mesoderm. An alternative, although not mutually exclusive, possibility is that a gradient of secreted noggin and/or chordin emanating from dorsal mesoderm attenuates BMP4 activity by regulating the amount of available BMP ligands in adjacent regions of the embryo. In fact, there is evidence that noggin can create a gradient of BMP4 activity, presumably because it antagonizes the

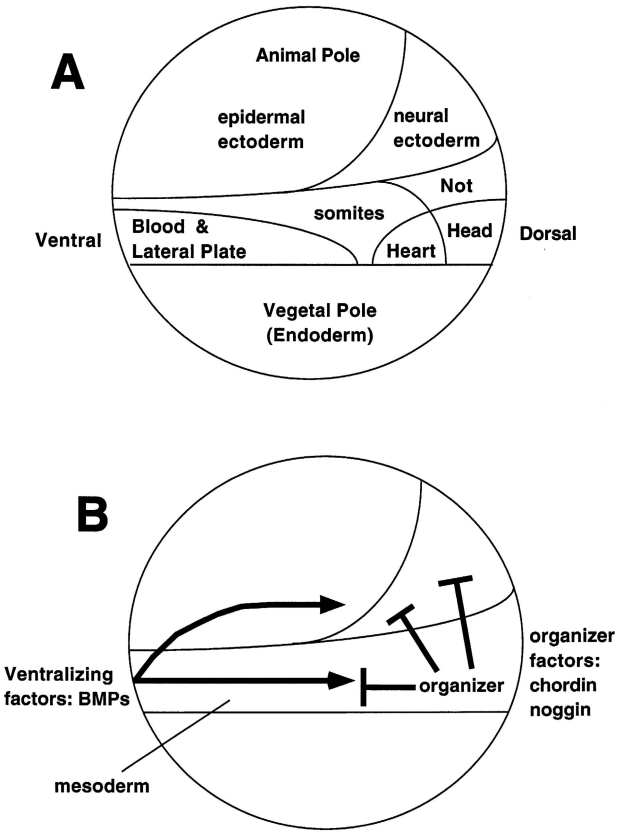


Figure 2 *A,* Fate map of *Xenopus* embryo. Mesodermal derivatives from the marginal zone are indicated. The dorsal-most mesoderm includes the notochord ("Not") and head mesoderm (including the prechordal plate mesoderm). *B,* Model of embryonic patterning in *Xenopus,* highlighting aspects involving TGF-β signaling. Inhibitory signals from the organizer (chordin and noggin) antagonize signals from BMPs, which are members of the TGF-β superfamily of cytokines. BMPs are distributed broadly throughout adjacent regions of the embryo, and their signals would block the induction of neural tissues and dorsal mesoderm if the signals from the organizer were not present.

action of BMP4. This graded activity, rather than the absolute concentration of BMP4 ligand, may be responsible for the dorsoventral patterning of the mesoderm (Dosch et al. 1997). By extension, chordin would be assumed to function similarly in dorsoventral patterning. It is important to keep in mind however, that the existence of a gradient of noggin or chordin protein has not yet been demonstrated in vivo.

The identification of Xolloid, the *Xenopus* homologue of the *Drosophila* gene *tolloid* (*tld*), provides an example of yet another level of extracellular regulation of BMP signaling (Piccolo et al. 1997). Xolloid and *tld* belong to the astacin family of metalloproteases, which also include BMP1. In Drosophila, *dpp* interacts genetically with *tld,* but the interaction is opposite to that of *sog,* in that *tld* is required in order to increase the activity of

dpp, whereas *sog* functions to inhibit *dpp* signaling (Marques et al. 1997). Similarly, in *Xenopus,* Xolloid can functionally antagonize chordin activity (Piccolo et al. 1997). For example, when injected ventrally, chordin can induce a secondary axis in *Xenopus* embryos (see sidebar), presumably by its ability to block BMP signaling. Xolloid can block the ability of chordin but, interestingly, not of noggin, to induce a secondary axis when they are coinjected ventrally. Biochemical data from both *Drosophila* and *Xenopus* have demonstrated that Xolloid and *tld* proteolytically cleave chordin and *sog,* respectively, thus preventing their ability to block BMP/*dpp* signaling (Marques et al. 1997; Piccolo et al. 1997).

Together, these data suggest the following model for dorsoventral patterning by the organizer-secreted molecules chordin, noggin, and Xolloid in *Xenopus* (fig. 2*B*). The ventralizing activity of BMPs is blocked dorsally within the organizer, by the binding of noggin and chordin to BMPs, thus preventing the BMPs from interacting with their receptors and functionally resulting in the specification of dorsal phenotypes. The action of chordin (and possibly of noggin) can be blocked extracellularly by proteolytic cleavage. The cleavage of chordin by Xolloid could serve to restrict the activity of chordin to the dorsal region of the embryo, by preventing active chordin from diffusing into adjacent regions of the embryo.

Future Directions

Because the number of identified TGF-β ligands far exceeds the number of identified receptors and signal transducers, it appears that cells do not have the luxury to respond to each ligand through a unique signal-transduction pathway; yet, cells still are able to respond to various TGF-β superfamily members in characteristic ways. Thus, cells must take advantage of the multiple combination opportunities available in this pathway, not only to generate specific responses to a range of TGF-β ligands but also to respond differently to the same ligand as the cellular context changes. As we have illustrated, the specificity of TGF-β signaling can be modulated at various levels, but fundamental questions remain regarding many of the molecular interactions and their role in signaling specificity. For example, although it is well recognized that TGF-β signaling requires an active heterotetrameric complex consisting of dimeric forms of type I and type II receptors, multiple forms of heterotetrameric receptor complexes potentially can be generated by various combinations of type I and type II receptors, as has been suggested for the case of the ActR-IIB receptor, which can function in association with either ActR-I or ActR-IB to confer different cellular responses (Carcamo et al. 1994; Armes and Smith 1997). Furthermore, although both type I and type II receptors exist as dimers, whether each receptor type forms homodimers exclusively or whether heterodimeric forms exist is not clear.

At the intracellular level, our understanding of the mechanisms by which smads control specific gene induction is limited. For instance, smads have been shown to have both intrinsic DNA-binding capability, as well as transcriptional transactivation activity (Whitman 1998), but they also have been shown to associate with various nuclear transcription factors, which together effect target-gene activation (Chen et al. 1996; Whitman 1998). Therefore, whether activation of smads is sufficient in itself to activate target genes in vivo is uncertain, as are the relative contributions of the smad DNA-binding domain and the transactivation domain, in the activation of target genes. Furthermore, Tsukazaki et al. (1998) recently showed that the regulation of the subcellular localization of the pathway-specific smad smad2 is important in the transduction of TGF-β signals. The FYVE-domain protein SARA (smad anchor receptor activation) functions in this localization by regulating the interaction of smad2 with the receptor complex at the membrane and smad4 in the cytoplasm. These authors propose that phosphorylation of smad2 by the type I receptor results in dissociation from SARA, with a concomitant formation of a smad2/4 complex and nuclear translocation. The mechanism by which smads are translocated to the nucleus, where they affect gene expression, remains unknown. These and other issues remain to be addressed, and we anticipate that, as in the past, the conclusions from work with *Xenopus* will dovetail with findings from other systems as these fundamental questions are answered.

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