Focus on TGF- β Signalling**The structural basis of TGF- β , bone morphogenetic protein, and activin ligand binding**S Jack Lin¹, Thomas F Lerch², Robert W Cook¹, Theodore S Jardetzky² and Teresa K Woodruff^{1,3}¹Department of Neurobiology and Physiology, ²Department of Biochemistry, Molecular Biology and Cell Biology,³Department of Medicine, Northwestern University, 2205 Tech Drive, Evanston, IL 60208, USA

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Abstract

The transforming growth factor- β (TGF- β) superfamily is a large group of structurally related growth factors that play prominent roles in a variety of cellular processes. The importance and prevalence of TGF- β signaling are also reflected by the complex network of check points that exist along the signaling pathway, including a number of extracellular antagonists and membrane-level signaling modulators. Recently, a number of important TGF- β crystal structures have emerged and given us an unprecedented clarity on several aspects of the signal transduction process. This review will highlight these latest advances and present our current understanding on the mechanisms of specificity and regulation on TGF- β signaling outside the cell.

Reproduction (2006) **132** 179–190**Introduction**

The transforming growth factor- β (TGF- β) superfamily is a large group of structurally related ligands that regulate a variety of cellular processes, including cell-cycle progression, cell differentiation, reproductive function, development, motility, adhesion, neuronal growth, bone morphogenesis, wound healing, and immune surveillance (reviewed in Kingsley 1994, Hogan 1996, Massague 2000, Attisano & Wrana 2002, Chang *et al.* 2002). The evolutionary significance of the family is highlighted by the conserved nature of the ligands across species. TGF- β orthologs are found from *Caenorhabditis elegans* through humans with equally conserved cell-surface receptors and signaling co-receptors. In mammals, the TGF- β superfamily can be further divided into three major subfamilies: TGF- β , activin/inhibin/nodal, and bone morphogenetic protein (BMP) (Dennler *et al.* 2002, Shi & Massague 2003). Each subfamily is composed of several isoforms that are associated with similar but non-overlapping physiological functions. Ligand members of the TGF- β superfamily consist of both homodimers and heterodimers, containing an ordered set of seven cysteine residues. Six cysteine residues form three intrasubunit disulfide bonds important for structural integrity, whereas the remaining cysteine residues form a disulfide bond with the other subunit to stabilize the dimer interface. Ligands are

expressed in tissue-specific patterns and can function in an endocrine, paracrine, and autocrine manner. Receptor specificity, tissue distribution, and expression levels may all affect the resultant cellular responses.

Cellular responses to most TGF- β ligands are transduced through interactions with two single membrane-spanning serine–threonine kinase receptors, called type I and type II receptors (Derynck 1994). Type I and type II receptors are glycoproteins of approximately 55 and 70 kDa respectively, which interact upon ligand binding. One unique feature of all type I receptors is the presence of a highly conserved TTS₂GS₂GS₂ motif in their cytoplasmic region, termed the GS domain, which plays a key role in regulating type I receptor kinase activity. The first identified receptor in the superfamily was the activin type II receptor (ActRII; Mathews & Vale 1991). Shortly later, a large class of serine–threonine receptors has been identified, with structural characteristics similar to the activin receptor. To date, four other mammalian type II receptors have been identified: ActRIIB (Attisano *et al.* 1992), AMHR-II (Baarends *et al.* 1994, di Clemente *et al.* 1994), T β RII (Lin *et al.* 1992), and BMPRII (Kawabata *et al.* 1995, Liu *et al.* 1995, Nohno *et al.* 1995, Rosenzweig *et al.* 1995). In addition, seven type I receptors termed activin receptor-like kinase (ALK)-1 to ALK-7 have also been cloned (Franzen *et al.* 1993, ten Dijke *et al.* 1993, 1994,

Tsuchida *et al.* 1993, 1996, Yamaji *et al.* 1994). Nonetheless, receptors within type I and type II classes differ in affinity and specificity, and may be expressed differentially to allow triggering of different components of the intracellular-signaling cascade. A major area of investigation is the structural basis of ligand–receptor interaction and cellular signaling.

A TGF- β ligand initiates signaling by binding to and bringing together type I and type II receptors on the cell surface to form a ternary holo-complex (Wells *et al.* 1999, Gilboa *et al.* 2000). The assembly dynamics leading to the holo-complex may differ between ligands. Some members of the BMP subfamily, the largest within the TGF- β ligands, can bind to either type I or type II receptor to stimulate the formation of holo-complex. Other TGF- β members, including TGF- β and activin, must bind to type II receptors, before type I receptors are recruited. Once the holo-complex is formed, the constitutively active kinase domain from the type II receptor transphosphorylates the GS domain of type I receptor, which in turn phosphorylates a number of intracellular mediator proteins known as Smads. There are eight distinct Smad proteins, constituting three functional classes: the receptor-regulated Smad (R-Smad), the co-mediator Smad (co-Smad), and the inhibitory Smad (I-Smad) (Massague *et al.* 2005). R-Smads (Smad1, Smad5, and Smad8 for BMP; and Smad2 and Smad3 for other TGF- β ligands) are directly phosphorylated and activated by type I receptor kinases. These R-Smads then undergo homotrimerization and form heterometric complexes with the co-Smad, Smad4. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear co-factors, regulate the transcription of target genes. The I-Smads, Smad6 and Smad7, negatively regulate TGF- β signaling by competing with R-Smads for receptor or co-Smad interaction and by targeting the receptors for degradation. A number of additional intracellular proteins regulate multiple steps within the intracellular signal transduction pathway.

Recently, a number of important TGF- β structures have been solved and have significantly advanced our understanding on the mechanism of specificity and regulation within the TGF- β -signaling cascade. In addition, a number of biochemical studies utilizing site-directed mutagenesis and domain-swap experiments have also delineated the role of specific amino acid residues in the structure-function dependencies for many of these TGF- β ligands and how they interact with their receptors. In this review, we will focus on the structural data pertaining to TGF- β signaling and regulation at the extracellular and cell-surface level.

Structure of TGF- β superfamily ligands and receptors

In 1992, the crystal structure of TGF- β 2 revealed a unique protein fold (Daopin *et al.* 1992, Schlunegger &

Figure 1 The TGF- β fold. A typical TGF- β monomer consists a cysteine knot motif with two pairs of antiparallel β -strands (fingers) extending from an α -helix ('wrist' region). The β -strands are curved to form both a concave and convex surface for receptor interaction.

Grutter 1992, Fig. 1). The general structure of the monomeric TGF- β ligand involves two pairs of antiparallel β -strands forming a flattened surface, projecting away from a long α -helix. Interestingly, one of the disulfide bonds of the core traverses through a ring formed by two other disulfide bonds generating what has been termed a 'cysteine knot' motif. The monomer has been described as a four-digit hand, with each β -strand being likened to a finger. At the N terminus, fingers 1 and 2 are antiparallel, with finger 2 leading to a general helix 'wrist' region, followed by antiparallel fingers 3 and 4 and the C terminus. Since then, the structures of several other TGF- β ligands have been elucidated, including TGF- β 1 (Hinck *et al.* 1996), TGF- β 3 (Mittl *et al.* 1996, Hart *et al.* 2002), BMP-2 (Scheufler *et al.* 1999, Kirsch *et al.* 2000b, Allendorph *et al.* 2006), BMP-7 (Griffith *et al.* 1996, Greenwald *et al.* 2003), BMP-9 (Brown *et al.* 2005), GDF-5 (Nickel *et al.* 2005, Schreuder *et al.* 2005), and activin (Thompson *et al.* 2003, 2005, Greenwald *et al.* 2004, Harrington *et al.* 2006), further confirming the identity of the TGF- β fold.

The active form of TGF- β is a dimer stabilized by hydrophobic interactions and usually further strengthened by an intersubunit disulfide bridge. Notable exceptions are BMP-15 and GDF-9, two structurally similar growth factors implicated in both oocyte maturation and follicular development (Shimasaki *et al.* 2004). These two ligands lack the cysteine, normally used to form the intersubunit disulfide bridge and may form as non-covalent homodimers or heterodimers with each other. Activin, inhibin, and a few BMP subunits have also been shown to form heterodimers. In fact, accumulating evidence suggests that several heterodimers, such as BMP-2/7 (Nishimatsu & Thomsen 1998) and BMP-4/7 (Aono *et al.* 1995, Suzuki *et al.* 1997) have more potent biological activity than the corresponding homodimers (Israel *et al.* 1996, Shimmi *et al.* 2005). Activin AB, a heterodimer of activin β A- and β B-subunits, has been

shown to signal through ALK7 in addition to ALK4, which is the type I receptor shared by all activin isoforms (Tsuchida *et al.* 2004). Equally intriguing is inhibin, a heterodimer consisting of a unique α -subunit and an activin β -subunit, which serves as a potent inhibitor of activin and BMP signaling (Lewis *et al.* 2000, Wiater & Vale 2003). The structural basis underlying this inhibin antagonism is presently not known.

Despite a common fold for all TGF- β monomers, striking differences can be observed by comparing the conformation of dimeric TGF- β ligands. Fig. 2A illustrates the ligand structures from each of the three major subfamilies: activin, BMP-7, and TGF- β 3. Most TGF- β ligands exhibit extended symmetric arrangements, like BMP-7. However, activin and TGF- β 3 have been shown to adopt alternative conformations, both resulting in the disappearance of the helical-wrist region. In one of the activin crystal structures available (Thompson *et al.* 2003), activin has a compact and folded-back conformation, pointing its fingers in nearly the same direction instead of outward. In the case of TGF- β 3, one monomer of the ligand rotates 105° upon binding to T β RII, resulting in an extended but open conformation (Fig. 2A and B). The dynamics of TGF- β 3 dimer have been characterized using NMR (Bocharov *et al.* 2002) and suggest that not only is the dimer less extended in solution than in the crystal structure, but also that the helical-wrist region is one of the most flexible regions of the protein. Flexibility at the dimer interface of TGF- β 3 provides a pivot point that the ligand can use to position

its receptors and may be mimicked in related ligands such as activin. Indeed, several activin crystal structures that are presently available reveal different wrist conformations, consistent with the idea of the wrist epitope being the most flexible part of the ligand (Fig. 2C–E).

The first TGF- β receptor structure solved was the ectodomain of ActRII (Greenwald *et al.* 1999). Since then, two other type II receptor ectodomains, T β RII and ActRIIB, have also been determined (Hart *et al.* 2002, Thompson *et al.* 2003). The general fold of the receptor resembles a class of neurotoxins known as the three-finger toxins, hence the receptor structure is referred to as having a three-finger toxin fold. This fold is comprised solely from β -strands stabilized by four disulfide bonds formed from eight conserved cysteine residues. Three pairs of antiparallel β -strands are curved to generate a concave surface for ligand binding. The only type I receptor structure presently available is the ectodomain of BMP-RIA (ALK3), which also exhibits the characteristic three-finger toxin fold (Kirsch *et al.* 2000b, Allendorph *et al.* 2006). Despite the common architecture and the cluster of conserved cysteine residues in the central β -sheets, very little sequence identity and no functional overlap exist between the two types of receptors.

Ligand:receptor interaction

The dimeric arrangement of TGF- β ligands suggests the formation of a complex with two type I and type II

Figure 2 Flexibility in TGF- β ligands. (A) Comparison of activin, BMP-7, and TGF- β 3 dimers. Monomers are colored red and blue in each of the structures and aligned by a single monomer (blue). (B) Comparison of free TGF- β 3 with receptor-bound TGF- β 3. The three different conformations of activin: (C) closed and folded-back conformation with a disordered wrist region (Thompson *et al.* 2003), (D) open and extended conformation (Greenwald *et al.* 2004), (E) open and extended conformation with a unique pre-helix, helix denoted with an asterisk (Thompson *et al.* 2005).

Figure 3 Receptor assembly dynamics. (A) Structure of BMP-2:BMP-RIA complex; BMP-RIA is shown in yellow. (B) Structure of TGF-β3:TβRII complex; TβRII is shown in green. (C) Structure of BMP-7:ActRII; ActRII is shown in green. (D) Comparison of different modes of receptor assembly for TGF-β, activin, and BMP.

receptors. This 1:2:2 ligand–receptor stoichiometry was predicted from a number of crystallographic structures, where the ligand is bound to either their high-affinity type I receptor at the ligand-concave surface (Kirsch *et al.* 2000b) or their high affinity type II receptor at the ligand-convex surface (Hart *et al.* 2002, Greenwald *et al.* 2003, 2004, Thompson *et al.* 2003, Fig. 3). Indeed, the predicted ternary-signaling holo-complex was recently confirmed with the crystal structure of BMP-2 ternary-signaling holo-complex (Allendorph *et al.* 2006).

The structure of the homodimeric BMP-2 bound to the ectodomain of its type I receptor, BMP-RIA, was the first TGF-β ligand:receptor complex solved (Kirsch *et al.* 2000b). In contrast to most other TGF-β ligands, BMP-2 can bind to type I receptor with high affinity. The structure reveals that the receptor binds to the ‘wrist’ epitope of the BMP-2 dimer and makes extensive contact with both BMP-2 monomers (Fig. 3A). These interactions are predominantly hydrophobic, with Phe85 of BMP-RIA playing a key role. The aromatic side chain of Phe85, with knob-into-hole packing, points into a hydrophobic pocket formed at the interface of the two BMP-2 monomers. Phe85 is preserved or replaced by a similar residue in all type I receptors except ALK1, and all residues that form the hydrophobic pocket in the dimeric BMP-2 are also highly conserved in all other TGF-β ligands. This suggests that hydrophobic knob-and-pocket binding is a characteristic feature at the ligand:type I receptor interface; however, other interactions are still required to define the exact specificity (Nickel *et al.* 2001, Harrison *et al.* 2003, 2004).

While the binding interface between type I receptors and their associated ligands appear to be common for all TGF-β family members, significant differences exist at the ligand:type II receptor interface. Unlike most BMPs, TGF-β and activin display a high affinity for type II receptors and do not stably interact with type I receptors. Structural analysis of TGF-β3 in complex with the ectodomain of TβRII reveals that binding occurs at the ‘fingertip’ region of the elongated ligand dimer (Hart *et al.* 2002, Fig. 3B). In contrast, the structure of BMP-7 and activin in complex with their high-affinity type II receptors, ActRII or ActRIIB, reveals a type II-binding site at the ‘knuckle’ region of the dimer (Greenwald *et al.* 2003, Thompson *et al.* 2003, 2004, Fig. 3C). Sequence analysis suggests that Mullerian inhibiting substance and its type II receptor, AMHR-II, may use a third unique binding surface (Greenwald *et al.* 2003), further highlighting the complexity in ligand binding to type II receptors. These observations identify different modes of receptor complex assembly and support the idea that type II receptors may play a more important role in determining specificity for the assembly of receptor-signaling complex than type I receptors.

Apart from crystallographic structures, our knowledge on the precise type I and type II receptor interaction sites has also been complemented with extensive mutagenesis, domain swap, and *in silico* studies. The mutagenesis approach has yielded some very accurate predictions. For instance, mutations in the helical wrist and concave finger regions of BMP-2 corroborates strongly with

Figure 4 Conservation of type I receptor-binding site. The concave finger region of activin (left) and BMP-2 (right) are shown. The BMP-2 residues shown to interact with BMP-RIA in the crystal structure and the corresponding residues on activin determined by mutagenesis studies are labeled. The side chains shown in dark blue represent residues contributing to the large hydrophobic patch responsible for type I receptor binding.

observations from the BMP-2:BMP-RIA crystal structure (Nickel *et al.* 2001). Using the same approach, mutagenesis has also localized the type II-binding site on BMP-2 to its convex finger region (Kirsch *et al.* 2000a), well before the BMP-7:ActRII crystal structure first provided a picture of the residues involved in the interaction. Similarly, crystal structures of activin or TGF- β in complex with its low-affinity type I receptors (ALK4 and ALK5 respectively) have not been unveiled. Nonetheless, there are considerable data on the location and residues of each ligand engaged in type I receptor interaction. *In silico* studies previously highlighted the position of aromatic and phenol rings as important determinants for receptor binding (Innis *et al.* 2000). The aromatic tryptophan residues integral to this site are highly conserved throughout the superfamily. Activin and BMP ligands share a number of identical or similar amino acid residues in this region (Fig. 4). Mutating one of the conserved tryptophan residues on activin produced a true activin antagonist able to bind only the type II receptor, and further mutations in this region have implicated a number of amino acids giving rise to a large hydrophobic patch on the concave finger region of activin (Cook *et al.* 2005). Also contributing to the type I interface is the wrist helical region; however, only large domain mutations in the wrist helix have led to the disruption of signaling, suggesting that ALK4 contacts both subunits of ligand concurrently (Muenster *et al.* 2005).

Different suggestions have been put forward to explain the two-step assembly of a functional signaling holo-complex for TGF- β ligands. For the most part, the cell membrane, allosteric ligand:receptor interactions, and direct receptor:receptor interactions could contribute to this sequential assembly of receptors. The cell membrane restricts the diffusional freedom of the ligand after it binds to its high-affinity receptor (type II receptor for TGF- β and activin; type I receptor for BMP). As a result, the ligand is effectively 'concentrated' to the cell surface and increases its likelihood of encountering and binding to its low-affinity receptor (Schlessinger *et al.* 1995).

Allosteric effects have also been proposed to explain the two-step assembly of receptors for some ligands.

The support for this idea comes from the fact that TGF- β and activin undergo large conformational changes when bound to their type II receptors, which could facilitate subsequent recruitment of type I receptors (Hart *et al.* 2002, Thompson *et al.* 2003, Greenwald *et al.* 2004). Molecular modeling of TGF- β 3:T β RII with BMP-2:BMP-RIA structure complexes also showed significant contacts between type I and type II receptors (Hart *et al.* 2002, Fig. 3D, top), suggesting that these could affect the assembly of the signaling complex. However, it is presently not known whether direct receptor-receptor contacts contribute significantly to the recruitment of low-affinity receptor. In addition, the BMP-2 ternary-signaling complex does not show the ectodomains of type I and type II receptors directly contacting each other (Allendorph *et al.* 2006, Fig. 3D, bottom). There remains some controversy, however, regarding the importance of co-operativity in TGF- β receptor assembly. A clear distinction should be made between pure avidity effects and co-operativity (reviewed in Sebald *et al.* 2004). Evidence available to support the apparent co-operativity (Greenwald *et al.* 2004) can also be explained with avidity effects. This avidity effect has been demonstrated by biosensor studies on TGF- β 3 (De Crescenzo *et al.* 2003). TGF- β 3 exhibits four orders of magnitude increase in affinity to its T β RII ectodomain when the receptor, instead of the ligand, is immobilized on the sensor. This apparent difference observed when one binding partner was immobilized instead of the other strongly suggests an avidity phenomenon caused by the stoichiometry of binding (De Crescenzo *et al.* 2003).

Extracellular antagonists

Extracellular antagonists of TGF- β signaling are important regulators of this signal transduction pathway. Antagonists such as noggin, follistatin, chordin/SOG, and DAN/Cerberus bind to ligands with high affinity and interfere with receptor engagement, thereby regulating many physiological responses. For example, in early embryonic development, antagonists produced in Spemann's Organizer are critical for the establishment

of proper BMP morphogen gradient (Harland & Gerhart 1997). It has recently been shown that the simultaneous depletion of noggin, follistatin, and chordin in *Xenopus* leads to a dramatic loss of embryonic dorsalization (Khokha *et al.* 2005), whereas single or double mutations on these antagonists only result in modest effects (Matzuk *et al.* 1995, Schulte-Merker *et al.* 1997, McMahon *et al.* 1998, Bachiller *et al.* 2000). Thus, these three TGF- β antagonists play an essential role in morphogenesis, with some overlap in their functions and ligand specificities. Presently, crystal structures for both noggin (Groppe *et al.* 2002) and follistatin (Thompson *et al.* 2005, Harrington *et al.* 2006) have been solved, making these two antagonists the most well understood with respect to their mechanisms of interaction and function.

Noggin is a 205 amino acid protein that is secreted as a glycosylated and covalently linked homodimer. Noggin is a BMP-specific antagonist protein that binds with high affinity to BMP-4, and with lower affinities to BMP-7, as well as homologs from *Drosophila* and *Xenopus* (Zimmerman *et al.* 1996). In the complex, noggin homodimers share a common two-fold symmetry axis with BMP-7, creating a large ringed structure (Fig. 5A and C). The N terminus of noggin is a 20-residue segment referred to as the 'clip,' which makes extensive contacts with BMP-7, followed by a 100-residue helical

domain that incorporates a highly basic heparin-binding segment for retaining noggin at the cell surface. The C-terminal half of noggin is composed of two pairs of antiparallel β -strands extending out from a cysteine-rich core, similar to the architecture of a TGF- β ligand.

On the other hand, follistatin exists in three different isoforms, with residues ending at 288 (FS-288), 305 (FS-305), and 315 (FS-315) (Schneyer *et al.* 2004). FS-315 is the dominant follistatin species found in circulation, while FS-288 is found primarily at the cell surface. This discrepancy can be attributed to their respective affinities for heparin: FS-288 binds strongly to heparin, while FS-315 binds with weaker affinity (Yamane *et al.* 1998). Although the structural basis for this discrepancy remains unclear, it has been suggested that FS-315 binds weakly to heparin because its elongated C-terminal tail, which is largely acidic, interacts and occupies the basic heparin-binding site on follistatin (Yamane *et al.* 1998). Unlike noggin, which binds solely to BMPs, follistatin binds with very high affinity to activin and with lower affinities to BMP ligands. In the complex, two follistatin molecules wrap around the activin dimers like two large C-clamps, forming head-to-tail contacts between their N- and C-terminal domains (Fig. 5B and D; Thompson *et al.* 2005). FS-288 contains a N-terminal domain (ND) and three follistatin domains (FSD1, FSD2, and FSD3), which

Figure 5 Mechanisms of antagonism by noggin and follistatin. (A) Structure of noggin:BMP-7 complex. The 'clip' segment is shown in yellow. (B) Structure of follistatin:activin complex. (C) and (D) Schematic of noggin and follistatin occluding both type I and type II receptor-binding sites on the ligand. Dotted lines in the follistatin complex represent contacts between follistatin ND and FSD3 domains that may lead to cooperative assembly of the neutralized complex. (E) Schematic of signaling complexes formed with type I and type II receptor binding. (F) Schematic of a potential asymmetric assembly of follistatin: BMP complex. BMP-RI can bind to the complex through the second type I receptor-binding site, but is incapable of signaling due to the absence of a nearby type II receptor.

are further divided into epidermal growth factor (EGF)- and Kazal protease inhibitor-like domains. Despite the striking difference in the structures between noggin and follistatin, these two antagonists share the same strategies by blocking type I and type II receptor binding sites on TGF- β ligands.

The surfaces of TGF- β ligands have two prominent hydrophobic patches, one on the concave type I receptor binding interface and the other on the convex type II receptor-binding interface (either the knuckle or the fingertip region). The hallmark of type I receptor binding is the presence of a conserved phenylalanine residue from the type I receptor inserted into a hydrophobic pocket on the ligand. In the case of noggin, the type I receptor-binding site on BMP-7 is occluded by a segment of the clip domain. Specifically, the hydrophobic ring of Pro35 from noggin inserts into a hydrophobic cleft on BMP-7, mimicking a similar insertion of Phe85 from BMP-RIA into the hydrophobic pocket on BMP-2. Follistatin takes a step further in mimicking type I receptor with its N-terminal domain, where Phe47 of FS-288 interacts with the hydrophobic residues on activin. Type II receptor binding is also characterized by extensive hydrophobic contacts between the receptor and the ligand. For noggin, the type II receptor-binding sites of BMP-7 are masked by the C-terminal half of the clip segment, as well as the distal tips of β -strands extending down from the noggin core. Similarly, follistatin buries approximately 75% of the type II receptor-binding surface on activin through a part of its FSD1 and the entire FSD2 domain.

Structural differences between noggin and follistatin have implications for potentially differing modes of TGF- β ligand inhibition. Activin binds follistatin with greater affinity than activin receptors (Hashimoto *et al.* 2000, Greenwald *et al.* 2004), hence follistatin almost irreversibly neutralizes activin by forming a high-affinity 2:1 follistatin–activin complex (Fig. 5D). The scenario is more interesting for BMP because it is antagonized by both follistatin and noggin. While noggin binds only to the ligand, follistatin has been shown to bind to both the ligand and the BMP-RI simultaneously (Iemura *et al.* 1998), suggesting that follistatin could inhibit BMP signaling by a unique mechanism. Type I receptors in a holo-complex may be activated preferentially by a proximal type II receptor located at the same end of the extended ligand fingers, thereby initiating signaling (Fig. 5E). In a 1:1 follistatin–BMP complex, BMP could interact with BMP-RI through its second type I receptor-binding site (Fig. 5F). This structure is feasible thermodynamically because the affinity of BMP for its type I receptor and follistatin are comparable (Iemura *et al.* 1998). As a result, BMP signaling may be disrupted not only by the formation of a 2:1 follistatin–BMP complex (similar to activin), but also in the form of an inhibited 1:1 follistatin–BMP complex containing BMP-RI. BMP signalling could be affected in these complexes by

blocking type II receptor recruitment, by altering signaling efficiency, and by sequestering BMP-RI. In contrast, noggin binds to BMP as homodimers, simultaneously blocking all receptor-binding sites. Compared to noggin's all-or-none approach, follistatin could have unique biological functionalities by engaging heterodimeric TGF- β ligands with two different binding affinities. Finally, whereas noggin serves as a BMP-specific antagonist, with the strongest affinity for BMP-4 and lower affinity for BMP-7, follistatin may provide a mechanism for more widespread inhibition for all BMP ligands by sequestering BMP-RI in a 1:1:1 follistatin:BMP:BMP-RI complex.

Membrane level regulation

Signaling by TGF- β ligands can be inhibited at the membrane level by the pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI). This 260 amino acid transmembrane protein shares 53% similarity with *Xenopus* BMP-RIA ectodomain and lacks an intracellular serine–threonine kinase domain. BAMBI has a short intracellular domain, which interacts with the intracellular domains of certain type I receptors for TGF- β s, activin, and BMPs to inhibit the formation of a signaling complex (Fig. 6A). While the extracellular domain does not have a high affinity for ligands, removal of this region creates a dominant negative protein, suggesting that the active region of the protein lies intracellularly (Onichtchouk *et al.* 1999).

Another form of inhibition comes from ligand sequestration by soluble forms of their high-affinity type I receptors (Fig. 6B). BMP-4 signaling can be inhibited by a soluble form of its high-affinity type I receptor (Natsume *et al.* 1997). TGF- β can associate with soluble type II receptors, blocking interactions with membrane-bound receptors (Fig. 6B, left; Lin *et al.* 1995, Tsang *et al.* 1995), and this inhibition is increased upon dimerization of the type II receptor (De Crescenzo *et al.* 2004). TGF- β 1 and TGF- β 3 can also be inhibited by their type I receptor, in a T β RII-dependent manner (Fig. 6B, right; Zuniga *et al.* 2005). In this scenario, the affinity for the type I receptor is increased by the formation of a ligand:type II receptor complex. Activin, like TGF- β , can interact with the ActRII ectodomain to inhibit signaling. Unlike TGF- β 1 and TGF- β 3, activin signaling is not significantly affected by the addition of ALK4 ectodomain (del Re *et al.* 2004b). This difference between TGF- β and activin reflects structural differences in their signaling complexes, in that TGF- β type I and type II receptors interact with one another upon holo-complex formation, whereas activin receptors interact with only the ligand (Fig. 3D). Thus, addition of the T β RII protein provides and expands the binding region for T β RI, while ActRII or ActRIIB does not change the surface area buried by ALK4.

Several ligands utilize co-receptors in mediating their signaling level. To date, at least three groups of

Figure 6 Modulators of TGF- β signaling at the cell surface. Ligand-signaling complexes can be inhibited by the pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI) or soluble forms of receptor extracellular domains. (A) BAMBI interacts with the intercellular domain of multiple type I receptor to block association of signaling receptors. (B) Left: soluble type II receptors inhibits signaling of TGF- β 1, TGF- β 3, and activin. Right: soluble type I receptors inhibit BMP-4 along TGF- β 1 and TGF- β 3 in complex with TGF- β type II receptor (T β RII). (C) Surface representation model of activin:ActRIIB. Activin monomers are colored blue and slate, ActRIIB in red. At their interface lie residues 94–107 (magenta), which convey independence from EGF-CFC co-receptors. (D) Inhibin and BMP-2, both requiring co-receptors for maximal activity, have lower affinity for their type II receptors than the co-receptor independent activin or BMP-7 (adapted from Greenwald *et al.* 2003, 2004).

co-receptors for TGF- β ligands have been identified: betaglycan and endoglin facilitate functioning of certain TGF- β ligands (TGF- β 2 and inhibin for betaglycan; TGF- β 1/TGF- β 3 for endoglin), EGF-cripto-FRL1-cryptic (CFC) proteins modulate signaling by Nodal and other related ligands, and DRAGON/repulsive guidance molecule-a (RGMa) proteins enhance signaling by some of the BMPs.

Betaglycan and endoglin are structurally related transmembrane proteins with a large extracellular region and a short cytoplasmic tail that lack any enzymatic activity (Lopez-Casillas *et al.* 1991, Cheifetz *et al.* 1992). An important function of betaglycan is to facilitate the binding of TGF- β and inhibin to their respective type II receptors, T β RII and ActRII/ActRIIB. This is particularly important for TGF- β 2 and inhibin, because both have only nominal intrinsic affinity for their type II receptor (Lopez-Casillas *et al.* 1993, Lewis *et al.* 2000). The extracellular domain of betaglycan consists of a

membrane-proximal uromodulin-like domain and a membrane-distal endoglin-like domain. While inhibin binds to only the uromodulin-like domain, TGF- β 2 can interact with either of the domains (Esparza-Lopez *et al.* 2001, Wiater *et al.* 2006). Indeed, similar to the high-affinity receptors discussed earlier, soluble betaglycan has been shown to inhibit TGF- β signaling (Lopez-Casillas *et al.* 1994, Vilchis-Landeros *et al.* 2001). Unlike the more broadly expressed betaglycan, endoglin is expressed primarily in vascular endothelial cells and syncytiotrophoblasts of term placenta (Lebrin *et al.* 2005). Also different from the monomeric betaglycan, endoglin is a disulfide-linked homodimer that binds ligand only when it is associated with T β RII. As a result, because T β RII binds more efficiently to TGF- β 1 and TGF- β 3, endoglin has higher affinities for TGF- β 1 and TGF- β 3 than TGF- β 2 (Letamendia *et al.* 1998, Barbara *et al.* 1999). Apart from type II receptor interactions, endoglin has also been proposed to facilitate TGF- β /ALK1

interaction over the more ubiquitous TGF- β /ALK5 in endothelial cells, leading to increased angiogenesis and cell proliferation (reviewed in Lebrin *et al.* 2005).

DRAGON and RGMA are GPI-linked proteins that bind to and enhance the signaling of BMP-2 and BMP-4, but not BMP-7 or other ligands (Babitt *et al.* 2005, Samad *et al.* 2005). In addition to binding to these two ligands, DRAGON and RGMA interact with several type I receptors, including ALK3 and ALK6. DRAGON also binds to ActRII, ActRIIB, and ALK2.

Members of the EGF-CFC family of proteins include the mouse and human Cripto and Cryptic, *Xenopus* FRL1, and Zebrafish one-eyed pinhead, and they are the required co-receptors for Nodal, GDF-1, Vg1, and GDF-3-signaling (Cheng *et al.* 2003, Babitt *et al.* 2005, Chen *et al.* 2006). EGF-CFC proteins contain a modified EGF-like domain followed by a unique CFC domain, as well as a GPI linkage at the C-terminus. Ligands requiring EGF-CFC co-receptors signal through the activin receptors ActRII/ActRIIB and ALK4. ALK4 interacts with the CFC domain (Yeo & Whitman 2001), while nodal appears to interact with the EGF-like domain of Cripto (Schiffer *et al.* 2001). Interestingly, while nodal and activin both use the same receptors, the prototypic EGF-CFC co-receptor Cripto appear to facilitate nodal signaling yet block activin signaling (Adkins *et al.* 2003, Gray *et al.* 2003). The basis for this difference in specificity is presently unknown and the mechanism of activin antagonism by Cripto remain controversial (Shen 2003). Previous work has localized the regions of activin that convey independence from EGF-CFC co-receptors to residues 94–107 (Cheng *et al.* 2004, Fig. 6C). They found that by inserting this region into Squint, a zebrafish homolog of nodal, the ligand could signal efficiently in the absence of its EGF-CFC co-receptor one-eyed pinhead. This region corresponds to the type II receptor-binding site on activin (Thompson *et al.* 2003, Greenwald *et al.* 2004), linking EGF-CFC dependence to type II receptor affinity. The finding that a myc-tagged ActRIIB is unable to pull down Vg1 or GDF-1 in the absence of an EGF-CFC protein supports this idea (Cheng *et al.* 2003).

Strengthening interactions between ligands and type II receptors is a common theme for TGF- β co-receptors. All ligands requiring co-receptors seem to have a lower affinity for type II receptors than ligands that signal independent of such co-receptors (Fig. 6D). It has been shown that in the absence of betaglycan, TGF- β 2 and inhibin have decreased affinities for type II receptors relative to their co-receptor independent counterparts TGF- β 1/TGF- β 3 and activin (del Re *et al.* 2004a, Greenwald *et al.* 2004). Similarly, BMP-2, which requires DRAGON/RGMA, has a lower type II receptor-binding affinity than BMP-7 (Greenwald *et al.* 2003), which signals independent of co-receptors.

There are several mechanisms by which these ligand:type II receptor interactions can be enhanced.

Because each co-receptor family is anchored to the membrane either by GPI linkage (DRAGON and EGF-CFC) or by a transmembrane domain (betaglycan and endoglin), these proteins may act by localizing their specific ligands to the cell surface, creating a higher local concentration of ligand about the type II receptor and thus allowing for a signaling complex to form. This would appear to be the case for betaglycan, as a soluble form of the protein acts to inhibit signaling (Vilchis-Landeros *et al.* 2001). The situation may be different for EGF-CFC proteins, such as Cripto, which has been shown to act in a non-autonomous fashion (Yan *et al.* 2002, Chu *et al.* 2005). EGF-CFCs may act to broaden the buried surface of the ligand when in complex with the type II receptors, similar to the proposed model of T β RII extending the binding region on TGF- β 1 and TGF- β 3 for their type I receptor (Zuniga *et al.* 2005). Cell-based assays should be sufficient in testing the first hypothesis of membrane localization by co-receptors, where structural characterization of co-receptor complexes would provide the majority of the information concerning the latter hypothesis. Experimentally defining the mechanisms by which these co-receptors modulate TGF- β signaling will elucidate how subtle structural differences lead to such a variety of cellular responses.

Conclusion and perspectives

Remarkable progress has been made on our understanding of TGF- β signaling in the past decade. An earlier focus on the cell biological characterization has now been complemented by biochemical and structural studies, giving rise to an unprecedented clarity in several aspects of the signaling transduction process. However, several important questions remain unanswered; for instance, future studies must address the basis of inhibin antagonism, different ligand specificities of EGF-CFC co-receptors, mechanism underlying receptor–ligand desensitization and trafficking, and how a bound receptor communicates with its cytoplasmic counterpart. The union of ideas and research between biochemical and structural studies with physiology will lead to an increase fund of knowledge about these critical ligands and new therapeutics that can regulate the myriad of TGF- β function in the body.

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