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Antagonism of Activin by Activin Chimeras

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Abstract

Activins are pluripotent hormones/growth factors that belong to the TGF- β superfamily of growth and differentiation factors. They play a role in cell growth, differentiation and apoptosis, endocrine function, metabolism, wound repair, immune responses, homeostasis, mesoderm induction, bone growth and many other biological processes. Activins and the related bone morphogenic proteins (BMPs) transduce their signal through two classes of single transmembrane receptors. The receptors possess intracellular serine/threonine kinase domains. Signaling occurs when the constitutively active type II kinase domain phosphorylates the type I receptor, which upon activation phosphorylates intracellular signaling molecules. To generate antagonistic ligands, we generated chimeric molecules that disrupt the receptor interactions and thereby the phosphorylation events. The chimeras were designed based on available structural data to maintain high affinity binding to type II receptors. The predicted type I receptor interaction region was replaced by residues present in inactive homologs or in related ligands with different type I receptor affinities.

Keywords

Activin; Bone Morphogenic Protein; TGF- β Superfamily; Chimera; Signaling; Antagonist

I. Introduction

Activins belong to the TGF- β superfamily of growth factors which control a variety of physiological functions such as cell growth, differentiation and apoptosis, endocrine function, metabolism, wound repair, immune responses, homeostasis, mesoderm induction, bone growth and many others (Massague, 1998; Risbridger et al., 2001; Schubert et al., 1990; Vale et al., 1990; Vale et al., 1986; Werner and Grose, 2003). The TGF- β family comprises at least 42 members in human (Shi and Massague, 2003) including activin, TGF- β , bone morphogenetic protein, growth and differentiation factor (GDF) and nodal proteins, which are all characterized by a distinct structural feature, namely a cysteine knot scaffold (Vitt et al., 2001).

Activins and most other members of the TGF- β superfamily are homo- or heterodimers consisting of two subunits, which are linked by a single covalent disulfide bond. Several subtypes of activin subunits are known and termed A, B, C, D and E. The dimeric activins are named after their subunits, i.e., activin A for an activin consisting of two A subunits, or

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AB for one consisting of one A and one B subunit. Proteins that were characterized biochemically include activin A, activin B, activin AB and activin C. It has not been unambiguously established whether proteins corresponding with activin D or E or other subunit combinations exist. The structures of several members of the TGF- β superfamily, including different TGF- β subtypes, activin-A, and several BMPs have been determined by X-ray crystallography and NMR procedures. In addition, for some of these ligands, structures in their bound state with receptor domains are available. Activins bind to two different subtypes of receptors termed type I and type II. Both receptor molecules are single transmembrane proteins. The N-terminal domain is engaged in ligand binding whereas the intra-cellular C-terminal domain exhibits serine/threonine kinase activities. In order to signal, activins initially bind to type II receptors with high affinity. The type I receptor is recruited into the complex and the constitutive kinase activity of the type II receptor phosphorylates residues in the intracellular kinase domain of the type I receptor, thereby activating it. Intracellular signaling molecules known as Smads are then phosphorylated and translocate to the nucleus where they modulate transcriptional events. BMPs are able to interact with activin type II receptors, in addition to BMP type II receptors, with high affinity. The specificity of the biological response is determined by the type I receptor which in turn interacts with a specific set of Smad signaling molecules. Inspection of the available structural and mutagenesis data allowed us to hypothesize that specific regions of activin and BMP are involved in interaction with either the type I or type II receptor. The goal of our investigation was the generation of activin mutants that would bind their type II receptor with near wild type affinity. By manipulating the residues involved in type I receptor interaction we aimed at generating ligands that could act as antagonists or be able to interact with different type I receptors thereby altering their biological activity.

II. The Activin/BMP Receptor System

A. Activins and their receptors

Activins were first isolated and characterized based on their ability to release the reproductive hormone FSH from anterior pituitary cells (Ling et al., 1986; Vale et al., 1986). Subsequently, activins were implicated in a variety of diverse biological activities. These include roles in nerve cell survival, wound healing and cell growth and differentiation. The major forms of activin are dimers of two closely related peptide chains, β a and β b, that form the active disulfide-linked proteins, i.e., activin A, activin AB and activin B. Other forms of activin chains, including the β c, β d and β e proteins are not widely expressed and their biological roles remain unclear. From radioactively labeled ligand crosslinking experiments it was known that activin bound to two receptor components. Based on their apparent molecular weights these had been termed type I for the lower molecular weight and type II for the higher one. The first member of this class of receptors from mammals, the activin type II receptor, was identified by expression cloning based on its ability to bind activin (Mathews and Vale, 1991). The protein was determined to be a single transmembrane molecule with an N-terminal ligand binding domain and an internal serine/threonine kinase domain. The type II receptor exists in two homologous forms, termed ActRII and ActRIIB. These are distinct gene products which function in a similar manner. Subsequently several type I receptors were identified by homology cloning and termed ALKs (Activin-receptor like kinases) (ten Dijke et al., 1993). Further studies showed that activin A predominantly interacts with ALK4 (also known as ActRIB), whereas activin B interacts with ALK7 (Bertolino et al., 2008; ten Dijke et al., 1994; Tsuchida et al., 1993). While activin binds its type II receptor with high affinity, it only interacts with the type I receptor after binding to the type II molecule. The type II receptor is a constitutively active kinase whereas the type I receptor's serine-threonine kinase activity is activated after being phosphorylated by the type II kinase. Phosphorylation of the type I receptor takes place in its unique GS domain near the intracellular juxtamembrane regions preceding the serine-threonine kinase domain.

The activated type I kinase then in turn phosphorylates Smad signaling molecules, specifically Smad 2 and Smad 3 in the case of the canonical activin type I receptor ALK4. Smad 2/3, which are also termed receptor activated Smads then form a complex with Smad 4 and trigger transcriptional activation events after translocation to the nucleus.

Activin signaling is highly regulated at multiple levels (Phillips, 2000). The extracellular binding proteins, follistatin and follistatin related protein which is encoded by follistatin related gene, bind the ligand with high affinity and render it unable to interact with its receptors (Tsuchida et al., 2000). At the cell surface, the pseudoreceptor 'BMP and activin membrane bound inhibitor' (BAMBI, (Onichtchouk et al., 1999)) interacts with several of the TGF- β family type I receptors and inhibits the formation of active receptor signaling complexes. Inhibin, the natural activin antagonist binds the activin type II receptors with high affinity once bound to betaglycan (Lewis et al., 2000) thus blocking the interaction with activin. Cripto, a member of the EGF-CFC (epidermal growth factor-Cripto/frl/cryptic) family, which attaches to the outer cell membrane and functions as a co-receptor for nodal signaling, also inhibits interaction of activin with its receptors (Gray et al., 2003). Further proteins that interact with activin type II receptor are ARIPs 1 and 2 (activin receptor-interacting proteins). These are PDZ (PSD-95/Disc-large/ZO-1) protein-protein interaction domain-containing proteins, that can either inhibit or enhance activin signaling, depending on the isoform expressed (Liu et al., 2006; Shoji et al., 2000). Further regulation occurs at the intracellular level of the Smad proteins, where inhibitory Smads (Smad 6, 7) disrupt TGF- β protein signal transduction. In the case of activin, Smad 7 has been shown to block signaling and to protect hepatocytes from activin-A induced growth inhibition (Kanamaru et al., 2001). Other proteins such as Smurf-type ubiquitin E3 ligases, Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998), and transcriptional co-activators and co-repressors like CBP, p300, c-Ski, and SnoN influence Smad activation, and thus influence activin-linked biologic effects (Chen et al., 2006).

B. BMPs and their receptors

Bone morphogenetic proteins (BMPs) were first identified as molecules that induce bone and cartilage formation in rodents (Wozney et al., 1988). BMPs are a large family (more than 20 members) with complex and diverse roles both in development and adult life (Matzuk et al., 1996; Whitman, 1998; Wozney et al., 1988). BMPs can be further classified into several subgroups, including the BMP-2/4 group, the BMP-5/6/7/8 group (OP-1 [osteogenic protein-1] group), the growth and differentiation factor (GDF)-5/6/7 group, and the BMP-9/10 group based on their biological activity (Kawabata et al., 1998). Many proteins of the BMP-2/4, OP-1, and BMP-9/10 groups induce formation of bone and cartilage tissues *in vivo*, while GDF-5/6/7 induce cartilage and tendon-like tissues, but not bone-like tissue. BMP-2, BMP-6, and BMP-9 were shown to be most potent in the induction of alkaline phosphatase activity and osteocalcin expression in the murine pluripotent mesenchymal cell line C3H10T1/2, and to induce differentiation of mesenchymal progenitor cells into osteoblasts, among 14 different BMPs and GDFs (Cheng et al., 2003).

BMPs, like activins, signal via type II and type I receptors (Nohe et al., 2002) and subsequently activate Smad proteins 1, 5, and 8, which in turn transmit signals into the cell nucleus (Heldin et al., 1997; Massague, 1998). Both types of receptors are needed to form a functional complex to initiate downstream signaling events. The oligomerization pattern of the BMP receptors was shown to be quite different from that of receptors used by other members of the TGF- β superfamily (Gilboa et al., 2000). The most prominent difference is the presence of BMP receptor oligomers, heteromeric complexes of BMPR-II/BMPR-IA or BMPR-II/BMPR-IB, at the cell surface prior to ligand binding. This finding suggests that the specific signals from such preformed complexes might be mediated by conformational changes within the subunits in the complex upon ligand binding. Interestingly, Nohe et al.

(Nohe et al., 2002) demonstrated that the mode of BMP receptor oligomerization determines which BMP signaling pathway is activated. In their studies, a measurable level of BMP receptors is found as preformed hetero-oligomeric complexes prior to ligand binding. However, majority of receptors were recruited into hetero-oligomeric complexes only after ligand addition, where BMP first bound to BMPR-II that then recruited a specific BMPR-I into the signaling complex. They reported that BMP binding to preformed receptor complexes activates the Smad signaling pathway, whereas BMP-induced recruitment of receptors activates a Smad-independent signaling pathway. In addition to the canonical pathway described above, several Smad-independent pathways have been shown to be activated including the p38 MAPK pathway (Derynck and Zhang, 2003; Iwasaki et al., 1999).

BMP type I receptors—BMPs can interact with two distinct type I receptors that are activin receptor-like kinases (ALKs). The type I receptors of BMPs are termed BMPR-IA (ALK-3) and BMPR-IB (ALK-6). BMPR-IA is widely expressed in various types of cells while expression of BMPR-IB shows a more restricted expression profile. Specificities of various BMPs binding to type I receptors are affected by type II receptors (Yu et al., 2005) and appear crucial for understanding the pleiotrophic effects of BMP action. BMP-2 and BMP-4 bind to BMPR-IA and BMPR-IB (ten Dijke et al., 1994), whereas BMP-6 and BMP-7 bind strongly to ALK-2, and weakly to BMPR-IB. GDF-5 preferentially binds to BMPR-IB, but not to other receptors (Nishitoh et al., 1996). BMP-9 and BMP-10 bind to ALK-1 and ALK-2 (Brown et al., 2005; David et al., 2007; Scharpfenecker et al., 2007). BMP type I receptors are shared by certain other members of the TGF- β family (Goumans et al., 2003; Oh et al., 2000). For example, MIS (Müllerian inhibiting substance) binds to ALK-2, BMPR-IA and BMPR-IB in the presence of its specific type II receptor, MISR-II.

BMP type II receptors—Although BMP type I receptors (ALK3, ALK6 and ALK2) are largely specific for BMP family, it is not true for type II receptors. BMPR-II binds only BMPs (Kawabata et al., 1998; Rosenzweig et al., 1995) (Liu et al., 2006) but activin type II receptors can bind both BMPs and activins (Yamashita et al., 1995). For example, BMP-3 can bind to both ActRII and BMPR-II and serve as modulators of growth factor activity during embryonic development. BMPs can thus bind to three type II receptors, i.e., BMPR-II, ActR-II, and ActRIIB, in mammals and these are widely expressed in various tissues. BMPR-II is specific for BMPs, whereas ActR-II and ActR-IIB can bind activins, myostatin, and BMPs. These type II receptors appear to bind most BMP ligands, and affect the binding preferences of BMPs to type I receptors (Yu et al., 2005). However, the majority of BMP signaling utilizes the BMPR-II receptor. BMPR-II has a unique, long C-terminal tail with 530 amino acids following the Ser/Thr kinase domain (Rosenzweig et al., 1995). The long form with the C-terminal tail is predominantly expressed in most types of cells, while the short form without the C-terminal tail may be expressed in certain types of cells as the tail region is shown to be not essential for BMP signaling in *Xenopus* embryo (Ishikawa et al., 1995).

Coreceptors for BMPs—Although type II and type I receptors are sufficient for transduction of intracellular signaling by BMPs, binding to receptors and signaling activity of certain ligands is regulated by coreceptors. For example, Glycosylphosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, including RGMa, b, and c, (RGMb and c are also known as DRAGON and hemojuvelin, respectively) act as coreceptors for BMP-2 and BMP-4, and enhance BMP signaling (Babitt et al., 2006; Babitt et al., 2005; Samad et al., 2005). In mouse pulmonary artery smooth muscle cells, BMP-2/4 signaling requires BMPR-II, but not ActR-II or ActR-IIB. However, cells transfected with RGMa use both BMPR-II and ActR-II for BMP-2/4 signaling, suggesting

that RGMA facilitates the use of ActR-II by BMP-2/4 (Xia et al., 2007). BMP-6 plays a key role in iron metabolism in hepatocytes. Upon BMP-6 stimulation in hepatocytes, hemojuvelin induces the expression of hepcidin, which in turn decreases iron absorption by the intestine and iron release from macrophages (Babitt et al., 2006) thus indicating that hemojuvelin functions as a signaling component in the BMP signaling pathway. Concurring this hypothesis, mutations in *Hemojuvelin* gene were identified in individuals with juvenile hemochromatosis (type 2A), which is characterized by accumulation of iron in various organs (Babitt et al., 2006) similar to the mutations in the *Hepcidin* gene in those humans. Another example is the transmembrane protein Endoglin, which is expressed in proliferating endothelial cells and other cell types, and binds to various ligands, including TGF- β 1/3, activin-A, and BMP-2/7 (Barbara et al., 1999). Although its function in TGF- β family signaling has not been fully determined, ectopic expression of endoglin results in inhibition of TGF- β -induced responses, whereas it enhances BMP-7-induced responses (Schermer et al., 2007). Mutations in the human *ENG* gene (encoding endoglin) result in hereditary hemorrhagic telangiectasia (HHT1, also known as Osler-Weber-Rendu disease) similar to those in *ALK1* (which induce HHT2), suggesting that they act in a common signaling pathway (Johnson et al., 1996; McAllister et al., 1994).

C. Structural studies

Available complex structures—Various crystal structures of ligands bound to their corresponding receptors' extracellular binding domains (ECD) have been resolved. So far, Activin bound to ActRIIB_{ECD} (Greenwald et al., 2004; Thompson et al., 2003), TGF- β 3 bound to T β RII_{ECD} (Hart et al., 2002) as well as a ternary complex of TGF β -3 bound to T β RI_{ECD} and T β RII_{ECD} (Groppe et al., 2008), BMP-7 bound to ActRII_{ECD} (Greenwald et al., 2003), BMP-2 bound to BMPRI-IA_{ECD} and ActRII_{ECD} (Allendorph et al., 2006) and BMP-2 bound to BMPRI-IA_{ECD} and ActRIIB_{ECD} (Weber et al., 2007), and GDF-5 bound to BMPRI-IB_{ECD} (Kotzsch et al., 2009) are available.

Architecture of complex structures—Not surprisingly, resolved complex structures reveal that TGF- β protein family members share common features with respect to ligand and receptor structure, as well as with regard to the overall architecture of ligand-receptor complexes. In each monomer of the ligand dimer, two pairs of antiparallel β -strands stretch out from the cysteine core of the dimer to form short and long fingers. The characteristic curvature of these fingers creates concave and convex surfaces on the ligand, which enable interaction with respective type I and type II receptor binding epitopes. At the base of the fingers, each monomer has an α -helix, which together with the pre-helix loop and the inner concave surface of the fingers of the other monomer, form the "wrist" region (Greenwald et al., 2004; Thompson et al., 2003). Each ligand dimer binds two type II receptors, and two type I receptors which leads to respective ternary signaling complexes.

Receptor-receptor interaction—Direct interaction between the receptor ECDs was not observed for BMP-2 and BMP type I and II receptors. In the BMP2:BMPRI-IA complex (Kirsch et al., 2000) no two atoms of BMPRI-IA-ECDs were closer than 35Å to each other. Also no contacts between ActRII-ECDs, and no contacts between ActRII ECDs and BMPRI-IA-ECDs were observed when models of BMP7:ActRII and BMP2:BMPRI-IA where combined to form the ternary complex of BMP7:ActRII:BMPRI-IA (closest distance: ActRII-ActRII, 83Å; BMPRI-IA-BMPRI-IA, 66Å). However, the gap between C-termini of ActRII-ECD and BMPRI-IA-ECD was 27Å only, leaving open the possibility of contact between transmembrane segments (Greenwald et al., 2003). A similar conclusion was drawn from the ternary complex structure of BMP2:BMPRI-IA:ActRIIB (Weber et al., 2007). No direct contact between ActRIIB-ECDs and BMPRI-IA-ECDs was observed, with the closest

proximity being 12Å. Distances between C termini were ActRIIB-ActRIIB, 85Å; BMPR-IA-BMPR-IA, 70Å; and BMPR-IA-ActRIIB, 40Å, excluding contacts of all four transmembrane helices, however, contact of BMPR-IA and ActRIIB transmembrane segments and thus an influence of transmembrane segment interaction on downstream signaling cannot be ruled out. Also in the Activin:ActRII complex no direct contact between the two receptor ECDs was observed with the closest proximity being 31Å (Thompson et al., 2003), however, whether or not receptor interaction between ActRII/ActRIIB and ALK4 occur, awaits structural data with the ALK4 extracellular domain. In contrast, TGF-β receptors type I and II ECDs are in close enough distance to make direct contact (Groppe et al., 2008; Hart et al., 2002). Based on this finding, Groppe et al. speculated that the markedly cooperative manner of the assembly resulting from the highly specific receptor-receptor interaction may contribute to a full biological response over a narrow concentration range, when compared to BMP signaling, which displays a wide dose-response range (Benchabane and Wrana, 2003; Groppe et al., 2008).

Ligand flexibility—BMP-2 and BMP-7 appear to be rather rigid ligands, with only minor conformational changes upon binding to their respective receptors. It was shown that the global fold of BMP-2 is not affected by binding to both, type I and type II receptors (Allendorph et al., 2006; Kirsch et al., 2000; Weber et al., 2007) with only small locally restricted changes in backbone and side chain conformations observed in the wrist and knuckle epitopes (Weber et al., 2007). Ligand binding to BMPR-IA caused an induced fit in the pre-helix region of BMP-2 (Pro48-Asn56). Also, binding of the BMP2:BMPR-IA complex to the type II receptor ActRIIB caused a small but significant reorientation of BMPR-IA (Weber et al., 2007), which did not occur in the complex of BMP-2 bound to BMPR-IA and ActRII (Allendorph et al., 2006). Therefore it has been speculated that the observed conformational change in BMPR-IA after type II receptor binding may be dependent on the nature of the type II receptor (Weber et al., 2007). In contrast, TGF-β3 and activin-A exhibit a high degree of flexibility with significant conformational differences observed between bound and unbound state (Greenwald et al., 2004; Hart et al., 2002; Thompson et al., 2003) (Harrington et al., 2006). NMR relaxation methods revealed an inherent flexibility in the TGF-β3 molecule with the open state predominating at pH 4 and below and the closed state at pH 5 and above (Bocharov et al., 2002; Mittl et al., 1996), which may contribute to a dynamic equilibrium between 'open' and 'closed' dimer architecture. The conformation also appears to depend on whether TGF-β3 is in complex with one pair of type II receptors (open conformation, (Hart et al., 2002) or in the full ternary complex (closed conformation (Groppe et al., 2008)). Similar explanations may be true for activins as well, since two different conformations of activin A have been described when bound to ActRIIB (Greenwald et al., 2004; Thompson et al., 2003). However, a ternary complex of activin bound to ActRII/ActRIIB and ALK4 has not been resolved yet. GDF-5 exhibits a somewhat intermediate flexibility when compared to the rigid BMPs, and the flexible activin-A and TGF-β3 (Kotzsch et al., 2009). Considering the observed differences in ligand flexibility within the TGF-β superfamily, it was speculated that dimer mobility within ligand-receptor complexes may play a role in quantitative aspects of signaling and SMAD protein phosphorylation (Kotzsch et al., 2009; Thompson et al., 2003), as it has been discussed earlier for the EPO receptor (Syed et al., 1998).

Binding epitopes—The ActRII (Allendorph et al., 2006; Greenwald et al., 2003) and ActRIIB (Greenwald et al., 2004; Thompson et al., 2003; Weber et al., 2007) binding epitopes are located in the convex curvatures of the ligand fingers, which is called the knuckle epitope. Interestingly, the ActRII binding site in the BMP7:ActRII complex (Greenwald et al., 2003) is slightly more shifted away from the fingertips, when compared to the ActRII binding site in the ternary complex BMP2:ActRII:BMPR-IA (Allendorph et al.,

2006). Therefore, it was speculated that the observed shift of the ActRII binding site is dependent on the ligand and thus may represent a mechanism for generating ligand specific receptor recognition (Weber et al., 2007). Ligand specific receptor identification was also observed with TGF- β 3, in which the TGFRII binding site is located in the finger tips (Groppe et al., 2008; Hart et al., 2002), and therefore significantly differs from the BMP-2, BMP-7, and activin-A knuckle epitopes that interact with ActRII and ActRIIB. The type I receptor binding epitope was assigned to the respective ligands' wrists in all complex structures involving type I receptors (Allendorph et al., 2006; Groppe et al., 2008; Kirsch et al., 2000; Kotzsch et al., 2009; Weber et al., 2007). A detailed list of the various ligands' amino acids involved in type I and type II receptor binding according to the resolved complex structures is depicted in the sequence alignment (Fig. 1). Additionally, numerous mutagenesis studies confirmed several of these amino acids to contribute to ligand receptor interaction (Fig. 1). With respect to type I receptor binding, eight missense mutations in GDF5 are known that cause skeletal malformation (Kotzsch et al., 2009), among those are three that cluster in the type I receptor binding epitope (R57L, deltaL56/S58T/H59L, and L60P). With the two latter, type I receptor binding was abrogated (GDF5 deltaL56/S58T/H59L) or greatly decreased (GDF5 L60P) in a binding assay using immobilized BMPR-IA and BMPR-IB-ECDs, whereas GDF5 R57L exhibited enhanced and unchanged affinity for BMPR-IA and BMPR-IB, respectively (Kotzsch et al., 2009). Another study using immobilized BMPR-IA and BMPR-IB ectodomains showed that BMP-2 W28F, D53A, Y103A hardly changed binding affinity for BMPR-IA and BMPR-IB, when compared to wt BMP-2, whereas BMP-2 D53P, S69R, and L51P mutants exhibited a reduced affinity for BMPR-IA and BMPR-IB, with the most drastic loss of binding observed for the L51P mutant. Additionally, on a functional level, BMP-2 L51P almost completely lost its ability to induce alkaline phosphatase activity in C2C12 cells (Keller et al., 2004). Looking at type II receptor binding, BMP-2 variants D34A, L90A, L100A, and S88A exhibited a significant reduction of binding affinity to their low affinity receptors ActRII- and BMPR-II-ECDs, as well as a relevant decrease in their ability to induce alkaline phosphatase in C2C12 cells (Kirsch et al., 2000), when compared to wt BMP-2. The strongest effect was observed with BMP-2 D34A, which after binding to its high affinity type I receptor, competed with wt BMP-2 for type II receptor binding, and thus acted as a BMP-2 antagonist in the before mentioned alkaline phosphatase assay using C2C12 cells (Kirsch et al., 2000). Furthermore, in an attempt to identify residues being responsible for high affinity binding of activin-A to ActRII/ActRIIB, BMP-2 residues were replaced by respective activin-A amino acids that were suspected to contribute to ActRII/ActRIIB binding. Indeed, the BMP-2 variant BMP-2 L100K/N102D (BMP-2 numbering) exhibited a strong increase in binding affinity for ActRIIB and to a lesser extent for ActRII, highlighting the relevance of these residues for ligand affinity for ActRII/ActRIIB (Weber et al., 2007). Earlier, BMP-2 variants S85R, E109R, and L100K were shown to increase receptor affinity for ActRII when compared to wt BMP-2 (Allendorph et al., 2006), and the K102E activin-A mutant was shown to lose its ability to bind ActRII and ActRIIB, and turned out to be inactive in a functional assay (Wuytens et al., 1999), underlining the importance of K102 in activin-A for high affinity binding to ActRII/ActRIIB.

III. Design of Chimeras

Activin mutants retaining high affinity for the type 2 receptor ActRII but losing the ability to signal in an activin like manner (e.g., by disruption of the type I receptor binding site) would be potential activin antagonists, and might reveal a general principle for the generation of desired antagonists of TGF- β superfamily proteins. Since numerous BMP-2 residues involved in ALK3 binding are located in the BMP-2 wrist (Allendorph et al., 2006; Keller et al., 2004; Kirsch et al., 2000) it was suspected that this region significantly contributes to type I receptor binding of activin as well. However, with respect to a recent finding showing

that the introduction of single point mutations in the activin wrist epitope does not have any significant effect on activin activity (Harrison et al., 2004) it was decided to generate activin chimeras with more severe mutations in the wrist. The biologically less active activin-C only shares 11 out of 32 amino acids in the wrist with activin-A which might contribute to its low activity and also to a lower affinity for ALK4 when compared to activin-A. Therefore, four mutants were constructed in which in each of them eight residues are changed from activin-A to activin-C (A/C 46–53, A/C 54–61, A/C 62–69, A/C 70–78), and one chimera with the entire wrist changed from activin-A to activin-C (A/C 46–78). Furthermore, structural data and mutagenesis studies described above not only proved the wrist epitope to be involved in type I receptor binding, but also lead to the assumption that the wrist epitope determines type I receptor specificity of respective ligands. Following this hypothesis, activin-A/BMP chimeras were constructed by exchanging the wrist region (residues 45–79 of activin A) to corresponding BMP-2 or BMP-7 residues. Subsequently, it was tested whether or not created chimeras are able to recruit BMP type 1 receptors.

IV. Assessment of binding and biological properties of chimeras

ActRII Binding

Based on the available data from previous crystal structure and mutagenesis studies, which showed that the ligands' type II receptor binding interfaces are located in the ligands' fingers (Greenwald et al., 2003; Kirsch et al., 2000; Thompson et al., 2003; Wuytens et al., 1999), the ActA/C and ActA/BMP chimeras were predicted to retain ActRII binding affinity, because both had been manipulated only in the wrist region. To prove this hypothesis, the chimeras' ability to displace ¹²⁵I-activin A from 293T cells transfected with mouse A χ tP χ II cDNA was assessed. Displacement curves revealed that EC₅₀ values of wt-activin-A for ActRII ranged between 90pM and 200 pM, and EC₅₀ values for ActA/C, ActA/BMP2 and ActA/BMP7 chimeras were 262–545 pM, 270 pM and 120 pM, respectively, indicating that the chimeras indubitably retained their affinity for A χ tP χ II (Fig. 2).

Activin-like bioactivity of chimeras

As the chimeras retained their type II binding as predicted, next the activin-like bioactivity of the ActA/C and ActA/BMP chimeras was determined. This was done by assessing their ability to activate an A3 Lux reporter gene in HEK 293T cells, in which wt-activin-A itself at a concentration of 20 nM led to a 32 fold induction of luciferase activity (EC₅₀ ~200 pM). A/C 46–53, 54–61, 62–69 and 70–78 (EC₅₀s ranging from 113–862pM) showed activities comparable to wt-activin-A (EC₅₀ ranging from 70–320pmol, Fig. 3A), however, the maximum activity of A/C 54–61, A/C 62–69 and 70–78 was somewhat reduced when compared to wt-activin-A. Only chimeras in which the entire wrist was changed from activin A to corresponding residues of activin C (A/C 46–78), BMP-2 (ActA/BMP2), and BMP-7 (ActA/BMP7) were devoid of significant activin-like bioactivity in concentrations of up to 40nM, suggesting that the chimeras lost their ability to signal in an activin-like manner (Fig 3B and 3C). Furthermore, the ActA/C chimeras retained their affinities for activin binding protein, Follistatin, which is in line with data published by Fischer et al. (Fischer et al., 2003), who generated a deletion mutant of activin A (activin des85–109) that does not bind follistatin, and thereby revealing that the C-terminus of activin-A is crucial for follistatin binding.

Antagonism

As the ActA/C 46–78 chimera and the ActA/BMP chimeras bind to A χ tP χ II and at the same time are devoid of activin-like bioactivity, they represent potential antagonists of proteins that signal via ActRII/ALK4 pathway like activins and myostatin (Rebbapragada et al.,

2003). Therefore, we determined the ability of the chimeras to block activin A and myostatin induced A3 Lux reporter in HEK 293T cells. The chimeras indeed inhibited A3 Lux reporter induction in a concentration dependent manner (Fig. 4). IC₅₀ ranges for the inhibition of luciferase activity induced by 100 pM activin A were 1–8 nM (Fig 4A), 1–10 nM (Fig 4B) and 4 nM (Fig 4C) for ActA/BMP7, ActA/BMP2 and ActA/C 46–78 respectively. Also, luciferase activity induced by 500 pM myostatin was reduced with IC₅₀ ranges of 1–5 nM (Fig 4A), 1–5 nM (Fig 4B) and 2.4 nM (ranging from 1–8 nM) for ActA/BMP7, ActA/BMP2 and ActA/C 46–78 respectively. The ActA/C 46–78 chimera could not block the luciferase activity induced by 50pM TGF- β (Muenster et al., 2005), which is in line with the fact that TGF- β acts via its own type II receptor, TGF- β RII, followed by the recruitment of ALK5. In contrast, both ActA/BMP chimeras were able to inhibit TGF- β induced luciferase activity (data not shown), which may be explained by BMP-like activity of the ActA/BMP chimeras (via introduced BMP wrist epitopes), thus leading to activation of Smads 1, 5 and 8, which are known to have a negative effect on TGF- β bioactivity (Goumans et al., 2003). The antagonistic properties of the ActA/C 46–78 and ActA/BMP chimeras were further substantiated through their significant inhibitory effect on basal and activin-induced FSH release from the mouse gonadotrope cell line L β T2 as well as from rat anterior pituitary cells in primary cell culture (Korupolu et al., 2008; Muenster et al., 2005).

BMP-like activity of ActA/BMP chimeras

Structural data and mutagenesis studies not only proved the wrist epitope to be involved in type I receptor binding (Allendorph et al., 2006; Keller et al., 2004; Kirsch et al., 2000) (Fig. 1 sequence alignment), but also lead to the assumption that the wrist epitope determines type I receptor specificity of respective ligands. Following this hypothesis, it was tested whether or not the ActA/BMP2 and ActA/BMP7 chimeras are able to recruit BMP type I receptors and signal in a BMP-like manner.

BMP-like activity of the ActA/BMP chimeras was assessed by characterizing their ability to activate BRE-Luc in HepG2 cells. HepG2 cells were used as they represent a relevant model (Duncan and Watt, 2001; Song et al., 1995) expressing both A α TPII and BMP type I receptors and thus is a BMP-responsive system (Song et al., 1995). In contrast to wt-activin-A, wt-BMP-2 and wt-BMP-7 stimulated BRE-Luc in HepG2 indicating that BRE-Luc activation is BMP-specific (Fig 5A and 5B). Wt-BMP-7 at a concentration of 40 nM led to a 23 fold BRE-Luc induction (EC₅₀, 5nM) whereas wt-BMP-2 at a concentration of 40 nM induced BRE-Luc 12 fold (EC₅₀, 4nM). The ActA/BMP chimeras stimulated the BRE-Luc in HepG2 cells with EC₅₀ values of 10 nM for ActA/BMP2 and 1 nM for ActA/BMP7 (Fig 5A and 5B). These data indicated that the ActA/BMP chimeras indeed signal in a BMP-like manner.

Further, ActA/BMP chimeras also exhibited BMP-like activity in the myoblastic cell line, C2C12. Under differentiating conditions, the ActA/BMP chimeras provoked a significant shift in the differentiation of C2C12 cells towards osteoblast-like cells expressing alkaline phosphatase. Also, both chimeras caused a substantial increase in Phospho-Smad1 but not Phospho-Smad2 in C2C12 cells, clearly establishing that the chimeras, when compared to wt-activin-A, have switched their binding affinities from the activin type I receptor ALK4 towards BMP type I receptors (Korupolu et al., 2008).

V. Summary, Conclusions and Future Directions

Signaling by ligands in the TGF- β superfamily is highly complex. Numerous ligands interact with a variety of receptors, which in turn activate diverse intracellular signaling molecules. The investigation of chimeric ligand molecules can shed a light on how specificity is achieved in such a system. We examined ligands of the activin/BMP subfamily. These

ligands exhibit promiscuity towards the type II receptors but recruit and activate specific type I receptors. Using guidance from available crystal structures, we were able to identify a region of the ligand molecule that is prominently involved in the interaction with type I receptors. When exchanging residues in this region in activin-A by ones present in the inactive activin-C subtype, the ligand still bound its type II receptor with near wild-type affinity, yet was unable to interact with type I receptors in a way that would lead to transduction of signal. Antagonism is thus achieved by occupying type II receptors and rendering them unavailable for wild-type activin binding (Fig. 6). Extending this model of activin/BMP subfamily signal transduction, we predicted that exchanging residues in the region by corresponding ones from BMP-2 or BMP-7, recruitment and activation of BMP specific type I receptors and concurrent Smad activation would occur. As demonstrated (Fig. 5), the BMP specific pathways are activated and phosphorylation of Smad 1 instead of Smad 2/3 occurs. Furthermore, as expected from the Activin A/C chimera the activin/BMP chimera acted as activin and myostatin antagonists as well. Altogether, data demonstrate that the wrist region of TGF- β protein family members significantly contributes to type I receptor specificity, and that manipulation of this region allows the construction of antagonists of respective wild-type ligands.

With respect to the implication of TGF- β protein family members in the pathogenesis of metabolic diseases such as diabetes and obesity, muscle wasting conditions like muscular dystrophy, sarcopenia, and cachexia, fibrosis, bone diseases such as osteoporosis, and cancer (Tsuchida et al., 2009), antagonists of TGF- β proteins represent highly interesting therapeutic options. Thus far, promising attempts to block signaling of TGF- β proteins for therapeutic interventions include type I receptor kinase inhibitors (ALK4, 5, and 7; (Ehata et al., 2007; Hjelmeland et al., 2004)), soluble forms of extracellular domains of ActRIIA (Deal, 2009; Pearsall et al., 2008) and ActRIIB (Lee et al., 2005) both fused with IgG-Fc. Furthermore, overexpression of activin-C (Gold et al., 2009) and ALK2 (Renlund et al., 2007) was demonstrated to antagonize activin effects *in vitro*.

With the approach to create TGF- β protein antagonists presented in this publication, further work to characterize such antagonists needs to be done. It remains to be established whether changing the entire wrist between different members of the TGF- β superfamily would allow the construction of desired type II receptor antagonists remains unanswered yet. Additionally, further improvement of antagonistic properties might be achievable by increasing the affinity of the chimera for ActRII (Wuytens et al., 1999). Ultimately, *in vivo* experiments need to be performed in order to check if the hypothesized biological effects of such antagonists will show.

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Figure 1. Sequence Alignment of activin A, activin C, BMP-2, BMP-7, GDF-5, and TGF- β 3

The mature, human activin A, activin C, BMP-2, BMP-7, GDF-5, and TGF- β 3 protein sequences were aligned using the Megalign program. Activin A wrist residues changed with activin C/BMP2/BMP7 are indicated by a black line above activin A; residues involved in type I receptor binding according to resolved crystal structures of respective ligand receptor complexes are shaded green (Allendorph et al., 2006; Keller et al., 2004; Kirsch et al., 2000; Kotzsch et al., 2009), and those involved in type I receptor binding according to mutagenesis studies are indicated by blue circles (Harrison et al., 2004; Keller et al., 2004; Kotzsch et al., 2009); residues involved in type II receptor binding according to resolved crystal structures of respective ligand receptor complexes are shaded red (Allendorph et al., 2006; Greenwald et al., 2003; Greenwald et al., 2004; Hart et al., 2002; Thompson et al., 2003), and those involved in type II receptor binding according to mutagenesis studies are indicated by red circles (Allendorph et al., 2006; Keller et al., 2004; Kirsch et al., 2000; Weber et al., 2007; Wuytens et al., 1999).

Figure 2. Competitive binding displacement curves

HEK293T cells were transfected with ActRII and subjected to competitive binding with unlabeled wt ActA, activin-A/C chimera or the different ActA/BMP chimeras as described (Korupolu et al., 2008; Muenster et al., 2005). Displacement curves are shown for activin-A/C chimera (A), ActA/BMP2 chimera (B) and ActA/BMP7 chimera (C). A standard displacement curve generated in the presence of various concentrations of unlabeled activin A is also shown (A, B and C). The values of each point on the graph is a mean $\pm$ S.D. of three measurements. Each experiment was repeated at least three times.

Figure 3. Activin-like bioactivity of chimeras

HEK293T cells were transfected with A3 Lux and β -galactosidase reporter plasmids as well as the transcription factor FAST2 and treated with increasing doses of wt activin A, activin-A/C chimera or ActA/BMP chimera, lysed, and assayed as described (Korupolu et al., 2008; Muenster et al., 2005). Dose response curves were generated for activin-A/C chimera (A), ActA/BMP2 chimera (B), and ActA/BMP7 chimera (C) as compared to wt activin A. Curves represent the luciferase activity normalized to β -galactosidase activity. Each point on the graph is the mean $\pm$ S.D. of three measurements.

Figure 4. Antagonism of activin A and myostatin by chimeras

HEK293T cells were transfected with A3 lux, β -galactosidase, FAST2, and treated with increasing doses of activin-A/C, ActA/BMP2, or ActA/BMP7 chimera in the presence of 100 pM wt activin A or 500 pM myostatin for 16–24 h. Cells were lysed and assayed for luciferase and β -galactosidase activities as described (Korupolu et al., 2008; Muenster et al., 2005). Dose-response curves activin-A/C + 100 pM wt activin A (*open triangles*) and activin-A/C + 500 pM myostatin (*closed downward triangles*) (A), ActA/BMP2 + 100 pM wt activin A (*open circle*) and Act/ABMP2 + 500 pM myostatin (*open downward triangles*) (B), and ActA/BMP7 + 100 pM wt activin A (*open square*) and ActA/BMP7 + 500 pM myostatin (*open diamond*) (C) were generated using Prism software. Curves represent the luciferase activity normalized to β -galactosidase activity. Each point on the graph is a mean $\pm$ S.D. of three measurements.

Figure 5. BMP-like activity of chimeras

HepG2 cells transfected with BRE-Luc and β -galactosidase were treated with increasing doses of wt activin A, wt BMP or ActA/BMP chimeras, harvested, and assayed as described (Korupolu et al., 2008). Dose response graphs for wt BMP-2 (closed circle), wt activin A (closed triangle), and ActA/BMP2 chimera (open circle) (A) and wt BMP-7 (closed square), wt activin A (closed triangle), and ActA/BMP7 chimera (open square) (B) were generated using Prism software. Curves represent the luciferase activity normalized to β -galactosidase activity. Each point on the graph is a mean $\pm$ S.D. of three measurements.

Figure 6. Mechanism of Activin Antagonism by chimeric ligands

Activin binds to its type II receptor and recruits the type I receptor into the complex. This results in phosphorylation and activation of the type I receptor's intracellular kinase domain. The activated type I kinase then phosphorylates Smad signaling molecules, which leads to downstream effects. The chimeric ligands described in this paper bind type II receptors with high affinity but are unable to recruit type I receptors thus disrupting the downstream phosphorylation/ activation events.