Structural Basis for a Functional Antagonist in the Transforming Growth Factor β Superfamily*

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Within the transforming growth factor β (TGFβ)3 superfamily, the agonist-antagonist relationship between activin and inhibin is unique and critical to integrated reproductive function. Activin acts in the pituitary to stimulate follicle-stimulating hormone, and is antagonized by endocrine acting, gonadally derived inhibin. We have undertaken a mutational analysis of the activin βA subunit to determine the precise structural aspects that contribute to inhibit antagonism of activin. By substituting specific amino acid residues in the activin βA subunit with similarly aligned amino acids from the α subunit, we have pinpointed the residues required for activin receptor binding and activity, as well as for inhibit antagonist of activin through its receptors. Additionally, we have identified an activin mutant with a higher affinity for the activin type 1 receptor that provides structural evidence for the evolution of ligand-receptor interactions within the transforming growth factor β superfamily.

The modern transforming growth factor β (TGFβ)3 superfamily consists of 42 encoded ligand subunits, five binding receptors (RII) that are constitutively active serine-threonine kinases, and seven signaling receptors (RI) that are essentially RII substrates (1). The ligands in this family are dimers that are disulfide linked in most, but not all, cases (GDF-9 and BMP15, for example) (2). A large family of ligands interacts with a limited array of receptor partners, which contributes to specificity of signaling at the cell surface.

The distinguishing characteristic of the TGFβ ligand subunits is six conserved cystine residues that form a core "cystine knot" motif analogous to that found in nerve growth factor, platelet-derived growth factor, and vascular endothelial growth factor (3, 4). Crystallographic studies indicate that the cystine pattern translates into a high degree of secondary and tertiary structural homology among subunits of the family (5–9). Ligands adopt a three-dimensional structure resembling a left hand, with a "wrist" domain and four "fingers" extended outward from the central cystine knot (Fig. 1). Similarities in receptor interaction sites on the ligands result from structural homology, and have led to the identification of the convex finger region of TGFβ superfamily ligands as the site for interaction with type II receptors, and the concave finger region as the type I receptor interaction site (6, 8–10).

Activin is a unique ligand within the TGFβ superfamily because it has a naturally occurring antagonist called inhibin. Inhibin is an endocrine hormone, also a member of the TGFβ superfamily, and blocks the paracrine actions of activin in a classical negative feedback loop (11, 12). One of the reasons that these ligands act as functional antagonists is their structural similarity. Activin is a dimer of two TGF-like β subunits (βA and/or βB), whereas inhibin is assembled from a βA or βB subunit and a distinct α subunit. The α and β subunits share less than 40% homology, but retain the conserved cystines. Connection of the two subunits through a disulfide bridge is necessary for production and activity of the antagonist.

At the surface of the pituitary gonadotrope, activin stimulates follicle-stimulating hormone (FSH) by binding with high affinity to the activin type II receptors, ActRII or ActRIB, inducing phosphorylation of the activin type I receptor, ALK4. ALK4 is a serine-threonine kinase receptor that phosphorylates cytoplasmic Smad2 and Smad3 transcriptional regulators (1). Inhibin binds ActRIB, presumably through its single β subunit, to block activin signaling through its cell surface receptor. Inhibin binds both ActRII and ActRIB, and possesses a higher affinity for the receptor in the presence of the accessory protein betaglycan (13).

To better understand the structure-function relationship between this agonist-antagonist pair, we generated a series of rationally designed point mutants that introduce an α subunit residue into the βA subunit and examined ActRIB binding characteristics and bioactivity. This study not only revealed the residues essential for activin binding to its receptors, but provided insight into the structural basis for inhibin antagonism of activin signaling.

MATERIALS AND METHODS

Mutagenesis and Protein Production—Activin A mutants were produced by the PCR technique according to the QuikChange procedure (Stratagene). Primers containing oligonucleotide substitutions encoding the codon for the desired amino acid were used to introduce mutations to a pcDNA3.0 plasmid containing the βA subunit of activin A. The mutation was verified by sequencing in an ABI3100 Capillary DNA Sequencer. Transfection of Chinese hamster ovary cells with plasmids encoding the proper mutation was performed according to the Lipofectamine 2000 (Invitrogen) method. Transfected cells were selected using G418 and carried in Dulbecco’s modified Eagle’s (DME)/F-12 media supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin. At confluence, media was exchanged for DME/F-12 serum-free media and grown for 5 days before collection. Following collection, serum-free media was dialyzed into 50 mM Tris, 150 mM NaCl, and
protein solution was concentrated by Amicon Ultra Centrifugal Filter Units (Millipore).

**Protein Concentration Determination by Fluorescence**—The concentration of wild type and mutant activin A solutions was determined by intensity of fluorescence using the ODDYSEY Imaging System (LI-COR). Western blot analysis was performed using a polyclonal activin βA antibody (provided by Dr. Wylie Vale, the Salk Institute), followed by an Alexa Fluor 680 goat anti-rabbit secondary antibody (Molecular Probes).

**Luciferase Assays**—Bioactivity of wild type and mutant activin A protein was determined by use of an LβT2 gonadotrope cell line stably transfected with the −338 region of the FSHβ promoter conjugated to a luciferase reporter. Cells were carried out as reported previously (14), and treated with 10 ng/ml mutant protein for 6 h at 37 °C in serum-free DME/F-12 without phenol red or serum, and incubated at 37 °C for 1 h. Plates were treated with 10 ng/ml of wild type or mutant activin A in Krebs-Ringer-Henseleit-BSA (128 mM NaCl, 5 mM KCl, 5 mM MgSO4, 1.3 mM CaCl2, 2 mM H2O2, 0.5 mM HEPES, 0.5% BSA) for 2 h at 37 °C. Cells were solubilized using RIPA buffer, and then treated with assay buffer (GME buffer, 16.5 mM KPO4, 2.2 mM ATP, and 1.1 mM dithiothreitol). Luciferase activity was measured for 30 s using an AutoLumat (Berthold Technologies Co.).

**Ligand Iodination and Binding Competition Assay**—Purified activin A ligand was iodinated using the lactoperoxidase method. 1 μg of ligand was diluted in 0.4 M sodium acetate, pH 5.6, immediately before addition of 10 μl of Na125I (MPBiomedicals). 5 μl of 1 unit/ml lactoperoxidase in 0.1 M sodium acetate, pH 5.6, was added, followed by addition of two 5-μl aliquots of diluted H2O2 with a 5-min incubation after each addition. The reaction was stopped by adding 272 μl of phosphate-buffered saline with 0.05% Tween 20.

COS-7 cells were carried in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Three days prior to the binding competition assay, cells were split to 2 × 10⁶ cells per 60-cm² plate. Twenty-four hours later cells were transfected with 10 μg of rat ActRIIB expression plasmid (provided by Dr. Kelly Mayo, Northwestern University) according to Lipofectamine 2000 (Invitrogen) method. Following overnight recovery in normal growth media cells were plated on 12-well plates. Following overnight recovery cells were incubated in Krebs-Ringer-Henseleit-BSA (128 mM NaCl, 5 mM KCl, 5 mM MgSO4, 1.3 mM CaCl2, 2 mM H2O2, 0.5 mM HEPES, 0.5% BSA) for 30 min at 37 °C. Cells were treated with appropriate solution of [125I]-activin A and/or competitor ligand, incubated at 4 °C for 3.5 h, then washed four times in ice-cold Krebs-Ringer-Henseleit-BSA before lysing with 0.5 M NaOH for 30 min at 4 °C. Cell pellets were counted in a Geiger counter to quantify radioactivity.

**Immunoprecipitation of Receptor Complexes**—Immunoprecipitation was performed on COS-7 cells transfected with rat ActRIIB-FLAG and rat ActRIIB-HA expression plasmids (provided by Dr. Kelly Mayo, Northwestern University) as described above. Cells split to 6-well plates were incubated in Krebs-Ringer-Henseleit-BSA for 2 h at 37 °C. Cells were solubilized using RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, and 5 mM EDTA). 1 μg of FLAG or HA monoclonal antibodies was added to each lysate for overnight incubation at 4 °C, followed by addition of 10 μl of protein A-Sepharose beads (Vector Labs) and a 2-h incubation at 4 °C. After washing beads and incubating in gel loading buffer at 90 °C, protein was electrophoresed on 12% SDS-PAGE gels. Detection of βA subunit was carried out as described above.

**Smad3 Phosphorylation Determination**—LβT2 cells split to 6-well plates were treated with 10 ng/ml of wild type or mutant activin A in DME/F-12 without phenol red or serum, and incubated at 37 °C for 1 h. Cells were lysed with lysis buffer containing protease inhibitors (Pierce), and 50 μl of lysate was run on 4–12% SDS-PAGE NuPage gels (Invitrogen). Western blot analysis was performed using phospho-Smad2 and -3 antibodies (Cell Signaling Technology, Inc.) at 1:500 dilution in TBS-Tween with 5% BSA. Detection of horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Zymed Laboratories Inc.) was performed according to the ECL Western Detection Kit (Amersham Biosciences).

**RESULTS**

The βA subunit, but Not the β Subunit, Binds ActRIIB—Unlike activin, TGFβ, and BMP, inhibin is a heterodimer of a β subunit and a unique α subunit. The α subunit retains seven of the conserved cysteines characteristic of ligands of the superfamily, but has significant differences in key topological domains thought to be important for ligand-receptor interaction (Fig. 1a). Previous studies demonstrated that inhibin has a greatly reduced affinity for the activin type II receptor in the absence of accessory proteins (13, 15). We used gel filtration chromatography of pre-assembled complexes to determine the molar ratio of ligand to receptor. The recombinant human ActRIIB extracellular domain (ECD) was produced in insect cells and purified by metal affinity chromatography as previously reported (9). Chinese hamster ovary cells were used to produce recombinant human activin A, and the ligand was purified using previously established methods (16) (Fig. 2a). Recombinant human inhibin A was a gift of Diagnostic Systems Laboratories, Inc. (Houston, TX). Activin- or inhibin-ActRIIB-ECD complexes were applied to a size exclusion column to determine the ratio of ligands and receptors in each complex (Fig. 2b). A complex at 58 kDa was resolved, representing a 26-kDa activin A molecule bound to two ActRIIB-ECD receptors (Fig. 2b, peak I). The inhibin A-ActRIIB-ECD complex eluted from the column at 45 kDa, reflecting a 32–34-kDa ligand bound to one receptor (Fig. 2b, peak II). Excess receptor eluted at a position equal to 18 kDa for both experiments (Fig. 2b, peak III). These data support those reported in recent ultracentrifugation experiments (5), and indicate that the α subunit is either unable to bind ActRIIB or binds weakly.

**Rationale, Design, and Synthesis of Activin-Inhibin Mutants**—The results of the chromatographic experiments described in the previous section prompted an analysis of the activin A-ActRIIB, activin A-ActRII, TGFβ3-βTBR1, and BMP2-BRIA structures (5–7, 9) to predict specific inhibin amino acids important to the mechanism of inhibin antagonism. ActRIIB binds to the convex surface of the finger domain of activin A. This region is characterized by a large number of hydrophobic amino acids (Fig. 1c) with side chains that pack against an equally hydrophobic-rich patch on the ActRIIB ECD. Compared with the TGFβ3-βTBR1 interaction, which occurs at the tips of the ligand fingers, the activin-ActRIIB binding region possesses a buried surface area nearly three times larger. Side chains for amino acids Phe117, Ile121, Ala123, His136, Ser139, Leu192, Tyr194, Ile199, and Lys200 point toward the receptor (Fig. 1c), and we replaced these residues with the corresponding α subunit residue, according to an alignment of the α and βA subunits, or with non-conserved amino acids (Fig. 1a). Of the 15 mutations produced in the ActRIIB binding region of activin (alignment of the subunits does not definitively specify Arg139 or Thr141 of the α subunit as being in the same site as Leu92 of the βA subunit, so mutations were made to both arginine and threonine) only substitution of phenylalanine at position 17 by asparagine appeared to alter processing. This mutation resulted in proteins of ~26, 28, and 30 kDa, presumably representing different glycoforms (Fig. 3).

The binding site for ALK4 on activin has not been determined. Modeling the receptor into the concave region of the activin βA subunit based upon the BMP2/BRIA crystal structure reveals 7 amino acids on
the βA subunit that form a hydrophobic pocket conductive to protein-protein interactions. Also, two charged residues at the edge of the hydrophobic pocket could have an impact upon receptor interaction (Fig. 1d). These residues are highly conserved in the inhibin α and BMP subunits (TABLE ONE). Mutations of Phe16, Ile23, Trp25, Trp28, Tyr35, Tyr93, Lys103, and Ile105 to glutamic acid, and of Asp96 to lysine, were introduced to the βA subunit.

Activin A mutants containing substitutions in ActRIIB and ALK4 binding sites were characterized as described under “Materials and Methods.” Wild type activin A produced using the same methods as used for production of mutant activin A served as a positive control for FSH bioassays, and competition studies. The bioassays utilized a mouse gonadotrope cell line, LβT2, and an activin-responsive reporter gene. Using this bioassay, we were able to achieve highly comparable lucifer-
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FIGURE 2. Determination of the molecular ratio of ActRIIB-ECD with activin A or inhibin A. Purified recombinant human ligand and receptor (a) were combined to form complexes of the ActRIIB extracellular domain with activin A (---) or inhibin A (--). Glycosylation of inhibin A and ActRIIB-ECD results in separation of two bands for each protein during electrophoresis. Proteins were eluted by size exclusion to determine molecular weight of the resulting complexes. The chromatogram of protein elution (b) indicates peaks corresponding to a ~58-kDa complex of activin A-ActRIIB-ECD (I), a ~43-kDa complex of inhibin A-ActRIIB-ECD (II), and excess ActRIIB-ECD at ~18 kDa (III).

FIGURE 3. Introduction of the glycosylation site on activin A. Immunoreactivity of concentrated media collected from cells transfected with empty pcDNA3 vector (pcDNA3) or with pcDNA3 vector containing wild type concentrated media collected from cells transfected with empty pcDNA3 vector.

TABLE ONE

| Comparison of BMP2, BMP7, βA, and α ligand subunits |
|----------------------------------|--------------|---------------|-------------------|
| BMP-2                           | Actinin βA   | Inhibin α     |
| Leu19                           | Leu45        | Leu35         |
| Val27                           | Leu90        | Leu42         |
| Trp29                           | Trp62        | Ile16         |
| Trp32                           | Trp55        | Trp47         |
| Tyr29                           | Tyr62        | Tyr94         |
| Tyr91                           | Tyr116       | Thr110        |
| Glu24                           | Asp119       | Asp106        |
| Lys101                          | Lys126       | Lys103        |
| Tyr103                          | Tyr128       | Ile105        |

Three concave surface activin A mutants, F16E, D96K, and K103E, exhibited normal bioactivity and binding characteristics (Figs. 4c and 6c), suggesting that alterations to the amino acid at these residues are not independently important to ActRIIB or ALK4 binding or biological activity. Like His36 and Tyr94 mentioned above, Phe16, Asp96, and Lys103 are located at the edge of the hydrophobic-rich pocket in the putative ALK4 binding site. Thus, the 9 amino acids whose mutations have little effect upon bioactivity and receptor binding, 8 are situated on the outer edge of the receptor binding sites.

Activin Mutants That Are Unable to Bind ActRIIB and Are Not Bioactive—The I30N, S90H, L92(A/R/T), and I100N activin A mutants were secreted by Chinese hamster ovary cells, yet none bound ActRIIB with normal affinity or exhibited biological activity comparable with wild type activin A (Fig. 4b and c). Thus, Ile30, Ser90, Leu100, and Ile100 are important to βA subunit interaction with ActRIIB. S90H and L92(R/T) are mutants in which an α subunit residue was substituted into the βA chain. The inability of these activin mutants to bind ActRIIB suggests that these residues are the key structural elements that define the functional difference between the βA and α subunits, and may be essential for inhibin antagonism of activin action through ActRIIB.

The substitution of a polar asparagine for Ile100 may disrupt the packing of receptor and ligand surfaces during interaction. As mentioned above, a phenylalanine substitution at this position most likely stabilizes the hydrophobic interaction, leading to a higher observed binding affinity. Consistent with this finding, substitution of Leu100 for a hydrophobic-rich region of ActRIIB and interacts with Val73 of the receptor, with a non-conserved polar side chain (asparagine) resulted in loss of binding and bioactivity.

βA subunit amino acids Trp28, Tyr35, and Tyr94 were predicted to bind ALK4. The α subunit has conserved residues in each of these positions, and the BMP2 amino acid sequence matches that of βA subunit exactly (TABLE ONE). Whereas each of these activin mutants is...
produced and secreted normally and can be detected by enzyme-linked immunosorbent assay, the substitution of glutamic acid residues results in proteins that have reduced affinities for ActRIIB and are not bioactive (Figs. 4c and 6). The disruption of interaction is evident in immunoprecipitation (IP) experiments (Fig. 6B), but W28E, unlike Y35E and Y93E, appears to have complete loss of interaction with ALK4. Because the side chains of these residues on the βA subunit point into the concave (putative R1) interaction site (Fig. 1d), non-conserved changes to glutamic acid may alter the conformation of the finger region, also disrupting ActRIIB binding.

**An Activin Type IIB Mutant with Normal Bioactivity—Substitution of the α subunit tyrosine for Lys102 of the βA subunit produced an activin A mutant with normal activity in both luciferase and FSH secretion assays (Fig. 4b and data not shown). Interestingly, K102Y only competes weakly with radiolabeled purified activin A for binding to ActRIIB expressed in COS-7 cells (Fig. 4c). To explore those contradictory results, we transfected LβT2 cells with an activin-responsive CAGA-luciferase reporter and a dominant negative ActRIIB receptor (DNRIIB), and treated the cells with activin A or K102Y mutant ligand (Fig. 5a). Introduction of the dominant negative receptor reduced lucif-
erase activity following treatment with wild type activin A. Luciferase activity following treatment with K102Y, however, was not significantly altered in the presence of DNRIIB.

Small interfering RNA technology was used to explore whether the K102Y mutant signals through the canonical activin pathway. Small interfering RNA directed toward the ALK4 receptor reduced luciferase activity in LβT2 cells treated with both activin A and K102Y mutant. Blocking ALK4 receptor with the ALK specific inhibitor, SB431542, leads to reduction in the phosphorylation state of Smad2 and -3 in cells treated with activin A or K102Y mutant (Fig. 5B). Small interfering RNA directed toward intracellular Smad3 also led to loss of bioactivity of both wild type and K102Y mutant activin A (Fig. 5C). K102Y interaction with ALK4 stimulated downstream phosphorylation of Smad2 and -3, and disruption of K102Y binding to the ALK4 inhibitor SB431542 resulted in loss of phosphorylation of both Smads as detected by immunoreactivity (Fig. 5D). Thus, the K102Y activin A mutant utilized the canonical activin signaling pathway.

To assess the direct interaction between K102Y and cell surface receptors, COS-7 cells were transfected with plasmids encoding FLAG-tagged ActRIIB and HA-tagged ALK4 receptor. Immunoprecipitation of receptor complexes from cells treated with wild type activin or the K102Y mutant indicated a reduced interaction of the mutant activin with ActRIIB compared with ALK4 binding (Fig. 5E). These data suggest that the K102Y activin A mutant may be functionally similar to several BMP ligands of the TGFβ superfamily, which have a higher affinity for their type I receptors.

Activin Mutants That Bind ActRIIB but Not ALK4—As mentioned above, Ile10 packs against a hydrophobic-rich region of ActRIIB, and interacts with Val73 of the receptor, and the non-conserved change to a polar side chain (asparagine) resulted in complete loss of binding. Insertion of a more conservative valine to mimic the α subunit resulted in binding to ActRIIB with wild type affinity. However, the I30V mutant
was not fully bioactive in any of the bioassays, suggesting that this mutant may affect downstream ALK4 binding and/or activation.

Ile23, Trp25, Trp28, Tyr35, Tyr93, and Ile105 at a treatment concentration of 10 ng/ml failed to induce luciferase production above background levels in activin-responsive cells (data not shown). Increasing the concentration 10-fold resulted in a higher luciferase response only in cells treated with I23E (Fig. 6a). We evaluated the phosphorylation of Smad3 to assess whether intracellular signaling events occurred with treatment of cells with the mutant ligands. An expected increase in the Smad3 phosphorylation state was observed for cells treated with 100 ng/ml I23E (Fig. 6a). This suggests that this mutant has a lower affinity for ALK4, but that the interaction is not completely disrupted.

IP experiments revealed that the W25E and I105E mutants bound to ActRIIB, but that binding of these mutants to ALK4 was disrupted (Fig. 6, b and c). Binding competition assays utilizing cells expressing only the type II receptor verified that the binding of W25E, and I105E, was similar to that of wild type activin A (Fig. 4c). We hypothesized that activin A mutants that exhibit normal binding to the type II receptor and disrupted ALK4 interaction would act as antagonists to wild type activin A signaling. The W25E mutant was purified, and its ability to disrupt activin A-stimulated luciferase production was assessed in LBT2 cells (Fig. 7). Two naturally occurring activin antagonists, inhibin A and follistatin, were also tested. At higher concentrations, W25E was able to disrupt activin A-stimulated luciferase activity better than inhibin A and comparable with Fst288.

**DISCUSSION**

The physiological role of activin is constantly expanding, from its originally described function in reproduction to current evidence of its
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FIGURE 7. Antagonism of activin A activity by W25E mutant. Luciferase response was measured in LfT2 cells treated with 200 \(\mu\)M wild type activin A alone, or in combination with increasing concentrations (0.2 to 50 nM) of purified W25E, inhibin A, or follistatin 288. W25E is able to significantly reduce luciferase production to background levels above 25 nM concentration of antagonist.

impact upon cell proliferation, changes in cell morphology, and its ability to recruit a wide number of cellular response factors (1, 17). Elucidation of the activin signaling mechanism, as well as its interactions with binding proteins, is also important for understanding the function of other ligands and receptors of the TGF\(\beta\) superfamily. An important difference between activin and the BMP and TGF\(\beta\) ligand subfamilies is the antagonistic association between inhibin and activin, which has been extensively studied in the hypothalamic-pituitary-gonadal axis. The currently accepted model of antagonism is one of competition by other ligands with mutation of the same residues to inhibin for activin type II receptors, primarily concentration based, and consistent with characterization of activin as a morphogen that directs cell and tissue development along a signaling gradient.

We expected dramatic differences in ligand-receptor interactions with the substitution of a tyrosine for Ala\(^{31}\) of the \(\beta\)A subunit. This amino acid is completely buried at the type II interface, and wild type level bioactivity of the A31Y mutant suggests that the aromatic cluster on the type II receptor surface (with which Ala\(^{31}\) interacts) is flexible enough to accommodate a large hydrophobic residue. The characteristics of this mutant may reflect a system responsive to the number of receptors occupied rather than the strength of receptor binding, and is consistent with characterization of activin as a morphogen that directs cell and tissue development along a signaling gradient.

The current study identified Ser\(^{50}\) and Leu\(^{92}\) as integral amino acids for the interaction of activin A and ActRIIB. Consistent with a previous study in which a L92A activin mutant was reported to have a reduction in bioactivity to 35% of wild type levels (10), these findings have established an important role for Leu\(^{92}\) in activin function. However, the current study also validates similarly aligned residues in the inhibin \(\alpha\) subunit as required for inhibin disruption of activin activity. The proximity of Leu\(^{92}\) to the pocket created by Tyr\(^{60}\), Trp\(^{78}\), and Phe\(^{103}\) on ActRIIB logically predicts that insertion of an ionic arginine side chain would be disruptive to the receptor interaction. Here, substitution of threonine or alanine for Leu\(^{92}\) also disrupted binding, reflecting the possible involvement of that residue in the inability of the \(\alpha\) subunit to bind ActRIIB.

The mutation of Ser\(^{90}\) may disrupt ligand-receptor interaction by a different mechanism from other mutants. Ile\(^{90}\), Leu\(^{92}\), and Ile\(^{100}\) are each positioned for optimal interaction with hydrophobic residues on the ActRIIB surface. In silico models of the \(\beta\)A subunit with a histidine at position 90 indicates a degree of steric hindrance resulting from the close proximity of Leu\(^{92}\) of the receptor. Although substitution of a histidine can also change the arrangement of the Ser\(^{90}\)-Lys\(^{102}\)-Asp\(^{104}\) triad, Ser\(^{90}\) and Asp\(^{104}\) appear to be optimally positioned for a hydrogen bond interaction. Depending on the rotameric positioning of the side chain in the S90H mutant, the histidine may not be able to stabilize the interaction between the positively charged amino group on Lys\(^{102}\) and the negative carboxyl group of Asp\(^{104}\). Regardless of the mechanism, Ser\(^{90}\) mutation to histidine reduces receptor binding. A recent mutation of this residue to alanine also led to reduction in activin bioactivity, although not to the extent observed in the current study (10).

Whereas non-conserved changes to Ile\(^{90}\) and Ile\(^{100}\) produced molecules unable to evoke an activin response or bind to ActRIIB, mutation of the same residues to inhibin \(\alpha\)-like amino acids had a limited effect on bioactivity, and bound receptor normally. The positions of both Ile\(^{90}\) and Ile\(^{100}\), close to Val\(^{73}\) and Val\(^{99}\) of the receptor, respectively, make the loss of activity upon insertion of an asparagine at either location seem logical. Mutation to valine or phenylalanine at these sites could hypothetically mimic the hydrophobic interactions observed with wild type activin A. Therefore, we conclude that those residues contribute to the interaction of ligand and receptor, but are not as integral to the disruptive nature of the inhibin \(\alpha\) subunit as Ser\(^{90}\) and Leu\(^{92}\).

The type II binding site on the \(\beta\)A subunit was also disrupted by substitution of a glutamic acid for tryptophan at position 28, and for tyrosines at positions 35 and 93. Each of the mutants is detected in both IP and enzyme-linked immunosorbent assay experiments, reflecting properly folded molecules. The residues mutated are highly conserved between activin A and BMP subunits, and the results presented here may be indicative of amino acids important for the structural integrity of the finger region of ligand subunits.

The K102Y activin mutant also had a low affinity for ActRIIB in binding competition assays, yet surprisingly exhibited activity close to that of wild type activin A in all of the bioassays utilized. Wuytens and colleagues (19) found that mutation of Lys\(^{102}\) of the \(\beta\)A subunit to glutamic acid or alanine resulted in reduced FSH production and Xbra...
expression by Xenopus animal caps, prompting the conclusion that the positive charge of Lys102 was important to biological activity and type II receptor interaction. Additionally, mutation of Lys102 to arginine produced a molecule with a comparable response to wild type activin. The authors briefly mention that the K102R mutant competed with wild type activin A binding to cells transfected with both receptors, and did not compete on cells transfected with ActRII alone (19). BMP2 and BMP4 have been shown to have a higher affinity for their type I receptor, BRIA, than for ActRII or BMPRII (20). Introduction of a tyrosine at Lys102 may be able to create a BMP-like molecule, and sequence alignment reveals a highly conserved hydrophobic leucine at that position in all members of the BMP subfamily (21).

We cannot discount the possibility that the K102Y mutation changes the TGFβ ligand dimer conformation and provides the impetus for type I receptor binding. The activin A-ActRIIB crystal structure provided clues for a change in ligand conformation, as activin was captured in a "folded back" position with the fingertip region of both subunits pointing in a similar direction. In contrast, the subunits of the BMP2 ligand are diametrically opposed in the BMP2-BRIA crystal structure. Mutation of Lys102 to tyrosine may result in an activin mutant with the capacity for stabilizing the movement of one βA subunit relative to the other. Whereas it is widely held that ALK4 receptor binding to activin requires prior ligand interaction with the type II receptors (22), this mutant may adopt a conformation less dependent upon activin A-ActRIIB complex formation. This does not necessarily remove the requirement for interaction with a type II receptor to promote intracellular signaling. Instead, the mutation may change the kinetics of ligand binding and holocomplex formation. Thus, our findings may reveal a structural evolutionary link between the older BMP ligands and the activin ligands.

The structure of BMP2 in complex with BRIA revealed an interaction of the receptor with the helical wrist region of one ligand subunit, and the concave finger region of the other subunit (7). Comparison of the structures and amino acid sequences of the BMP2 and activin βA subunits infers a similar mode of interaction of activin A with ALK4. Whereas the inhibin βA subunit possesses the structural aspects necessary for disruption of ActRIIB binding, it may also interact with ALK4 via conserved residues on the concave surface of the α subunit and the helical region of the βA subunit. The inhibin-like mutation at Lys102 also provides precedence for α subunit binding to ALK4. As mentioned above, data suggest that inhibin antagonism of activin is dependent upon the interaction of the antagonist with the type II receptor. Prior experiments have provided evidence that inhibin, in the presence of activin receptors, yields complexes that only contain ActRII or ActRIIB (13, 18). However, amino acid sequence similarities between the activin receptors, yields complexes that only contain ActRII or ActRIIB. As mentioned above, data suggest that inhibin antagonism of activin is dependent upon the interaction of the antagonist with the type II receptor. Prior experiments have provided evidence that inhibin, in the presence of activin receptors, yields complexes that only contain ActRII or ActRIIB (13, 18). However, amino acid sequence similarities between the activin receptors, yields complexes that only contain ActRII or ActRIIB.
phobicity, with charged residues Asp96 and Lys103 located on the periphery of the site.

The current mutational analysis has provided three key insights into the structural aspects of the activin βA subunit. Substitution of inhibin α subunit residues at positions 90 and 92 of the βA subunit disrupts normal activin interaction with its type II receptor. This will prove important for developing peptides that mimic the α subunit structure, and potentially slow unregulated activin activity in diseases such as premature ovarian failure. Also, substitution of βA subunit residues with those of the α subunit uncovered an activin mutant with increased affinity for the type I receptor, ALK4. Whether this mutant reflects a BMP-like mutation, or confers an affinity of inhibin for ALK4, remains to be determined. It would be interesting, although, to ascertain whether this mutant is able to recover phenotypes observed in type II receptor knock-out mice. Finally, we have developed mutations in the concave region of the βA subunit that identify a novel region of interaction with ALK4. In addition to producing a physiologically important activin antagonist, this discovery provides the impetus for exploring a new mechanism of activin down-regulation resulting from inhibin interaction with activin receptors at the surface of the pituitary gonadotrope.

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