Reconstitution and Analysis of Soluble Inhibin and Activin Receptor Complexes in a Cell-free System*

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Activins and inhibins compose a heterogeneous subfamily within the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors with critical biological activities in embryos and adults. They signal through a heteromeric complex of type II, type I, and for inhibin, type III receptors. To characterize the affinity, specificity, and activity of these receptors (alone and in combination) for the inhibin/activin subfamily, we developed a cell-free assay system using soluble receptor-Fc fusion proteins. The soluble activin type II receptor (sActRII)-Fc fusion protein had a 7-fold higher affinity for activin A compared with sActRIIB-Fc, whereas both receptors had a marked preference for activin A over activin B. Although inhibin A and B binding was 20-fold lower compared with activin binding to either type II receptor alone, the mixture of either type II receptor with soluble TGF-β type III receptor (TβRIII; betaglycan)-Fc reconstituted a soluble high affinity inhibin receptor. In contrast, mixing either soluble activin type II receptor with soluble activin type I receptors did not substantially enhance activin binding. Our results support a cooperative model of binding for the inhibin receptor (ActRII+sTβRIII complex) but not for activin receptors (type II + type I) and demonstrate that a complex composed of activin type II receptors and TβRIII is both necessary and sufficient for high affinity inhibin binding. This study also illustrates the utility of this cell-free system for investigating hypotheses of receptor complex mechanisms resulting from crystal structure analyses.

Activins A and B, members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors, have a variety of actions in both embryonic and adult tissues, including tissue fate determination in vertebrate embryos, regulation of follicle-stimulating hormone biosynthesis in pituitary gonadotrophs, and regulation of gametogenesis in ovaries and testes (reviewed in Ref. 1). Although five vertebrate activin genes have been identified (βA–βE), only activins A (βA subunit homodimer) and B (βB subunit homodimer) have experimental evidence supporting biological roles in vivo (2), although a role for activin C was recently proposed in the liver (3). When examined in vitro, recombinant activins A and B appear to have similar biological activities (4, 5). However, in vivo, disruption of the mouse βA gene results in early neonatal lethality with craniofacial defects and lack of whiskers, suggesting that activin A is critical for normal mammalian development (6). In contrast, disruption of the βB gene is not lethal but results in fertility defects in homozygous knockout females related to inability to nurse newborn pups (7). Interestingly, replacement of the βA mature coding sequence with that from the βB gene, resulting in activin B production wherever activin A or B would be synthesized in wild-type animals, rescues many of the skeletal abnormalities of the activin A knockout but still results in fertility defects and growth retardation, indicating that activin B cannot completely compensate for activin A even when expressed in an identical fashion (8, 9). It therefore appears that the two activins subserve at least some non-overlapping functions in the adult, although the mechanism whereby this differential activity is manifested remains unclear. One possible explanation is that distinct subsets of activin receptors may differentially bind activins A and B, at least in some tissues.

The wide distribution of activin A and B expression in the adult suggests that they primarily act as autocrine or paracrine signals. On the other hand, inhibin, a heterodimeric molecule composed of one of the activin βA or βB subunits linked to a distantly related α subunit to produce inhibin A or B, respectively, was originally discovered as an endocrine regulator of follicle-stimulating hormone biosynthesis, being synthesized primarily in gonadal tissues and acting on pituitary gonadotrophs (reviewed in Ref. 10). Specific assays for human dimeric inhibins A and B indicate that they are differentially produced during the menstrual cycle and that alterations of inhibin concentrations are inversely associated with changes in follicle-stimulating hormone concentrations in a variety of fertility abnormalities in both men and women (reviewed in Ref. 1). In addition, animal studies have indicated that inhibin may also have autocrine/paracrine activities within the gonads, including influencing steroidogenesis and gametogenesis (11). However, disruption of the inhibin α subunit in the mouse, which would be expected to delete both inhibins A and B, results in the appearance of aggressive gonadal tumors that kill most animals before reproductive defects can be fully assessed (12). Interestingly, activin A and B levels are severely exaggerated animals before reproductive defects can be fully assessed (12). Interestingly, activin A and B levels are severely exaggerated

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1 The abbreviations used are: TGF-β, transforming growth factor-β; ActRI, activin receptor; TβR, TGF-β receptor; ECD, extracellular domain; α, soluble; PBS, phosphate-buffered saline; BMP, bone morphogenetic protein.
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binds directly to one of two identified type II receptors, ActRII or ActRIIB, which recruits one of two type I receptors, ActRIA or ActRIIB (also known as Alk2 (activin-like kinase-2) and Alk4, respectively). Following ligand binding, the type I receptors are phosphorylated by the type II receptors to initiate the intracellular signaling cascade (reviewed in Refs. 14 and 15). Although inhibin can bind to activin type II receptors, this binding is at least 10-fold lower compared with activin (16) and thus does not appear to be sufficient to account for the endocrine activity of inhibin. This situation was recently clarified when a high affinity inhibin receptor comprising ActRII and the TGF-β type III receptor (TβRIII; betaglycan) was proposed (17). Presumably, when ActRII is complexed with inhibin and TβRIII, ActRII is inhibited from associating with activin and ActRI, thereby disrupting activin signaling. However, because these studies were conducted in transfected cells, they could not exclude the participation of other as yet uncharacterized cell-surface proteins in this binding complex. Thus, determination of the affinity and specificity for each possible activin- or inhibin-binding complex would benefit from pure soluble receptors that could be analyzed individually or in complexes, thereby clarifying results from cell-based experiments.

We have recently shown that a purified fusion protein containing the TβRII extracellular domain (ECD) linked to human Fc is a useful tool to explore receptor complex specificity and ligand binding affinity (18). We have now created soluble (s) ActRII and ActRIB ECD-Fc fusion proteins (sActRII-Fc and sActRIIB-Fc, respectively) and explored their affinity and specificity for activin and inhibin. In addition, purified sTβRII-Fc fusion protein in combination with sActRII-Fc or sActRIIB-Fc formed high affinity inhibin-binding complexes in solution, confirming earlier cell-based studies. Finally, in contrast to TGF-β receptors, we did not observe any potentiation of activin binding to sActRII-Fc or sActRIIB-Fc by soluble activin type I receptors, consistent with the non-cooperative model proposed from the crystal structure of ActRIIB (19). Thus, our studies using purified soluble receptors demonstrate the specific requirements for high affinity binding of activins and inhibins to different complexes of activin type II and type I and TGF-β type III receptors, without the confounding variables that whole cell systems necessarily confers.

MATERIALS AND METHODS

Production of Soluble Receptors—The ECDs of ActRII (residues 1–135) (20) and ActRIIB (residues 1–134) (21) were amplified by PCR and cloned in-frame with the Fc tag of a modified Signal pIgplus vector (R&D Systems, Minneapolis, MN) as described previously for sTβRII-Fc (18). The ECD of TβRII (22) was similarly cloned, and the glycosaminoglycans were eliminated by site-directed mutagenesis at S353A and S464A. The Fc tail of Signal pIgplus contains a free cysteine so that the resulting proteins are secreted as dimers (see “Results”).

The receptor-Fc plasmids were transfected into 293 cells, which were cultured in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen). All transfections were performed with Lipofectamine 2000 (Invitrogen). Stably transfected cells were selected with 1 mg/ml G418 and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% ultra-low IgG fetal bovine serum (Invitrogen) in 175-cm² multiwell flasks (TPP, Transadsingen).

Recombinant receptor-Fc fusion proteins were purified by protein A affinity chromatography. Tissue culture medium (1 liter/batch) was adjusted to pH 8.2 and applied to a HiTrap rProtein A FF column (Amersham Biosciences AB, Uppsala, Sweden) previously equilibrated with 10 mM phosphate-buffered saline (PBS). After protein loading, the column was washed with PBS to remove nonspecifically bound proteins and eluted in 100 mM glycine buffer (pH 3.0). Eluted fractions were immediately neutralized by addition of 1 M Tris-HCl (pH 9.0). The concentration of eluted protein was determined by bovine serum albumin protein assay (Pierce) and confirmed by amino acid analysis. Protein purity was assessed by 4–12% SDS-PAGE using precast gels (In-vitrogen) followed by silver staining (Bio-Rad) according to the manufacturer’s protocol.

Receptor-Fc proteins were also analyzed by Western blotting. Each preparation was separated on 4–10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). After transfer, the membrane was washed with PBS supplemented with 0.1% Tween 20 (PBST) and blocked overnight in 8% dry milk in PBST. The membrane was incubated with goat anti-AcActRII, anti-AcActRIIB, or anti-TβRIII antibody (R&D Systems) or with anti-human Fc antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and then with horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence detection was performed with Renaissance Western blot chemiluminescent reagent (PerkinElmer Life Sciences). Mouse ActRIA-Fc and ActRIB-Fc proteins were purchased from R&D Systems.

Idination—Human activins A and B (R&D Systems) were iodinated by the lactoperoxidase method as purified by electrophoresis as described previously (5). Inhibins A and B (R&D Systems) were iodinated using the reduced chloramine-T method described previously for TGF-β (23) as adapted for inhibin (24) and purified by gel filtration chromatography.

Ligand Binding Assays—Soluble recombinant human receptors were diluted in Tris-buffered saline/casein blocking buffer (BioFX, Owings Mills, MD) and incubated A-containing protein A-coated plates (Pierce) at 4 °C. Plates were then washed with wash buffer (BioFX) and blocked for 2 h at room temperature with Tris-buffered saline/casein blocking buffer. For competition binding assays, fixed amounts of radioligand (50,000–10,000 cpm) was added to the receptor-coated plates together with increasing amounts (2 pM to 500 nM) of homologous or heterologous nonradioactive ligands as described under “Results” and incubated overnight at 4 °C. After washing three times with wash buffer with wash buffer, wells were separated and counted in a γ-counter.

To allow inhibin receptor proteins to form complexes prior to binding assays, these experiments were performed with soluble binding assays. sActRII-Fc or sActRIIB-Fc with or without (control) sTβRII-Fc proteins (as indicated in the figures) were diluted in Tris-buffered saline/casein blocking buffer and incubated overnight with a fixed amount of radiolabeled activin or inhibin and increasing amounts (2 pM to 500 nM) of unlabeled inhibin at 4 °C. The samples were then plated on protein A-coated plates, incubated for 1 h at room temperature, washed, and counted as described above. The same protocol was employed to perform ActRII-ActRI complex binding experiments, except that iodinated activin A or B was used as radioligand.

Assessment of Biological Activity—Human embryonic kidney 293 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Transient transfections were performed in 24-well trays using Effectene (QIAGEN Inc., Valencia, CA) and a total of 200 ng of DNA, including 80 ng of the Smad-responsive reporter CAGA-Luc (a gift from Dr. S. Dunnler) (25), 20 ng of pRL-TK (Promega Corp., Madison, WI), and 100 ng of nonspecific plasmid DNA. Following 16 h of post-transfection incubation, cells were treated with or without 0.1% Triton X-100, 0.1% sodium deoxycholate, or 0.1% bovine serum albumin for 6 h at 23 °C. Cleavage was verified by resolving 0.1 g of the treated material on a Ready-Gel as described previously (23) as adapted for inhibin (24) and purified by gel filtration chromatography.

Removal of the Fc Tag—To determine whether the presence of the Fc tag altered biological activity, sActRII-Fc or sActRIIB-Fc was preincubated with or without equal amounts of Factor Xa (New England Biolabs Inc., Beverly MA) in 20 μl of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM CaCl₂, and 0.1% bovine serum albumin for 6 h at 23 °C. Cleavage was verified by resolving 10 ng of the Factor Xa- or mock-treated receptors on a 12% Ready-Gel (Bio-Rad) under reducing conditions, followed by Western blotting as described above.

To determine whether the presence of the Fc tag altered biological activity, sActRII-Fc or sActRIIB-Fc (0.5 μg) was incubated with or without 0.1 μg of Factor Xa (New England Biolabs Inc., Beverly MA) in 20 μl of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM CaCl₂, and 0.1% bovine serum albumin for 6 h at 23 °C. Cleavage was verified by resolving 10 ng of the Factor Xa or mock-treated receptors on a 12% Ready-Gel (Bio-Rad) under reducing conditions, followed by Western blotting as described above.

Inhibin reporter assay—To determine whether the presence of the Fc tag altered biological activity, sActRIA-Fc or sActRIIB-Fc (0.5 μg) was incubated with or without equal amounts of sActRIA-Fc or sActRIIB-Fc (5 μg) was treated with or without 0.1 μg of peptide G [N-glycosidase F (New England Biolabs Inc.) at 37 °C for 1 h. The completion of the deglycosylation reaction was confirmed by resolving 0.1 μg of the treated material on a Ready-Gel as described above and staining with Silver Stain Plus (Bio-Rad) following the manufacturer’s protocol.
Characterization of Soluble Activin Receptors—Recombinant sActRII-Fc and sActRIIB-Fc fusion proteins purified from conditioned medium were analyzed by silver staining of SDS-polyacrylamide gels and found to be >95% pure. sActRIII-Fc ran as a single band of 55,000 Da, whereas sActRIIB-Fc ran as a doublet of 53,000 and 57,000 Da, probably due to glycosylation, whereas ActRII-Fc is a single band at 55,000 Da. Both proteins were >95% pure. B, purified sTβRIII-Fc (TBRIII-Fc; betaglycan), subjected to SDS-PAGE under reducing conditions and silver staining, shows a single band at ~110,000 Da. This protein was >95% pure. C, soluble fusion proteins were analyzed by SDS-PAGE under reducing conditions and Western blotted with anti-Fc antibody. Protein bands identical to those in A and B were identified, except only the larger band of sActRII-Fc was stained, perhaps reflecting a sensitivity to glycosylation in this antibody. D, the same proteins as in C but run under nonreducing conditions show that all three soluble fusion proteins exist in solution as dimers. Interestingly, the sActRIIB-Fc dimer was slightly smaller than the sActRII-Fc dimer.

**RESULTS**

**Characterization of Soluble Activin Receptors**—Recombinant sActRII-Fc and sActRIIB-Fc fusion proteins purified from conditioned medium were analyzed by silver staining of reduced SDS-polyacrylamide gels and found to be >95% pure. sActRII-Fc ran as a single band of ~55,000 Da, whereas sActRIIB-Fc appeared as a doublet of ~53,000 and 57,000 Da (Fig. 1A). sTβRIII-Fc was also a single band under reducing conditions with an apparent molecular mass of ~120,000 Da (Fig. 1B); and consistent with deletion of glycosaminoglycans, none of the typical heterogeneity of molecular mass was observed. To characterize the multimeric nature of these recombinant proteins, they were analyzed by Western blotting with anti-human Fc antibody under reducing (Fig. 1C) and nonreducing (Fig. 1D) conditions. Although the relative molecular mass of each receptor-Fc fusion protein agrees with that obtained from the silver-stained gel when reduced (Fig. 1C), the fusion proteins are clearly dimeric under nonreducing conditions, most likely via disulfide bonding between the Fc chains.

**Affinity and Specificity of the Soluble Activin Receptor Fusion Proteins**—The affinities of sActRII-Fc and sActRIIB-Fc for [125I]activin A or B were determined by Scatchard analysis, with representative experiments shown in Fig. 2 and means of at least three replicates shown in Table I. The affinity of sActRII-Fc for activin A was surprisingly high, averaging 49 pM (Table I) compared with 150–400 pM for the natural membrane-anchored receptor (28) and 2–7 nM for the soluble receptor ECD with no tag (29). In addition, this affinity was >60-fold greater than for activin B (3.2 nM) (Fig. 2 and Table I). In contrast, the affinity of sActRIIB-Fc for activin A (326 pm) was ~7-fold lower than that of sActRII-Fc (Table I), but closer to that observed for the membrane-bound form of 100–380 pM (30, 31). Like sActRII-Fc, the affinity of sActRIIB-Fc for activin B was lower than for activin A (1.9 nM) but higher than the affinity of sActRIIB-Fc for activin B (Table I). These results demonstrate that the activin type II receptor-Fc fusion proteins are capable of high affinity binding to activin and are selective for activin A over activin B. Moreover, sActRII-Fc binds activin A at an affinity surpassing that of the intact membrane-anchored form of ActRII.

The relative potencies of activins A and B compared with inhibins A and B were investigated for sActRII-Fc. When activin A was the radioligand (Fig. 3A and Table I), unlabeled activin A was the most potent competitor (ED₅₀ = 0.73 ± 0.09 ng), whereas the potency of activin B was ~4-fold lower (ED₅₀ = 3.26 ± 1.29 ng). Inhibins A and B were 20-fold (ED₅₀ = 15.8 ± 0.39 ng) and >30-fold (ED₅₀ = 23.7 ± 2.59 ng) less potent, respectively, than activin A in competing with radiolabeled activin A. When radiolabeled activin B was examined in this system (Fig. 3B and Table I), unlabeled activin A was 8-fold more potent compared with unlabeled activin B (ED₅₀ = 3.75 ± 1.02 ng), indicating that the difference in binding activity between activins A and B for each radioligand was not due to alterations from the iodination process, but was more likely related to aspects of ligand structure that are differentially recognized by sActRII-Fc and sActRIIB-Fc. Consistent with our observations using radiolabeled activin A, inhibins A and B were >20-fold less potent than unlabeled activin A in competing with radiolabeled activin B. These results indicate that sActRII-Fc is selective for activin A, followed by activin B (~30-fold less) and inhibins A and B (20–30-fold less), similar to what was reported for the membrane-bound form of the receptor (28).

**Biological Activity of Soluble Activin Receptors**—To investigate the ability of the soluble activin receptor-Fc proteins to modulate activin biological activity, we employed a previously described activin reporter assay consisting of the CAGA activin-TGF-β luciferase reporter transfected into 293 cells. As shown in Fig. 4A, treatment with 0.18 nM activin A resulted in >30-fold stimulation of reporter activity. This stimulation was inhibited dose-dependently by sActRII-Fc, which was 2-fold more active than the high affinity activin-neutralizing protein FST288. Interestingly, sActRIIB-Fc was ~5-fold less effective in inhibiting activin A-stimulated reporter activity compared with sActRII-Fc. When the cells were treated with 0.12 nM activin B, the neutralization activity of the soluble receptors was similar to that observed for activin A (Fig. 4B), except that FST288 was less effective in inhibiting activin B compared with activin A as observed previously (32). These results indicate that the soluble receptor-Fc fusion proteins are effective inhibitors of activin action in vitro. Moreover, given that both soluble activin receptors were purified to similar degrees (Fig. 1A), the difference in inhibitory potency of sActRII-Fc versus sActRIIB-Fc for activins A and B in this biological assay likely results from the difference in activin binding affinity (Table I).

**Effect of the Fc Tag and Glycosylation on ActRIIA/B Biological Activity**—To determine whether the differences in the affinity and biological potencies of sActRII-Fc and sActRIIB-Fc reflect actual structural differences in the binding domains of the fusion proteins or differential effects of the Fc tag, we examined the effect of removing the tag on biological activity.

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**Fig. 1. Analysis of soluble receptor ECD fusion proteins.** A, purified sActRIIB-Fc and sActRII-Fc fusion proteins were analyzed by SDS-PAGE under reducing conditions and silver staining. ActRII-Fc ran as a doublet of 53,000 and 57,000 Da, probably due to glycosylation, whereas ActRIII-Fc is a single band at 55,000 Da. Both proteins were >95% pure. B, purified sTβRIII-Fc (TBRIII-Fc; betaglycan), subjected to SDS-PAGE under reducing conditions and silver staining, shows a single band at ~110,000 Da. This protein was >95% pure. C, soluble fusion proteins were analyzed by SDS-PAGE under reducing conditions and Western blotted with anti-Fc antibody. Protein bands identical to those in A and B were identified, except only the larger band of sActRII-Fc was stained, perhaps reflecting a sensitivity to glycosylation in this antibody. D, the same proteins as in C but run under nonreducing conditions show that all three soluble fusion proteins exist in solution as dimers. Interestingly, the sActRIIB-Fc dimer was slightly smaller than the sActRII-Fc dimer.

**Data Analysis**—Binding data were analyzed by the NIHRIIA program (26) for parallelism and relative potency (ED₅₀). For homologous assays, dissociation constants were determined by Scatchard analysis using the LIGAND program (27). Each curve was also analyzed by Prism (GraphPad Software, San Diego, CA), which gave nearly identical results. Data shown in tables and all dissociation constant estimates were derived from the LIGAND analysis, whereas Figs. 2 and 7 were produced by Prism. Results shown in figures are representative experiments, and data tables represent the means ± S.E. of at least three replicates. Significance of differences in radiolabeled activin binding to mixtures of activin type II and type I receptors was assessed by Student’s t test with p < 0.05 used to indicate significance. Results from bioassay experiments are expressed as the means ± S.E. of at least three replicates.
Treatment with Factor Xa for 24 h released the Fc tag from both activin receptor fusion proteins as shown by the faster mobility of the ECD following SDS-PAGE of the cleavage products (Fig. 5A). However, this treatment resulted in no detectable enhancement of biological activity since there was no increase in sActRIIB-Fc bioactivity when tested at the same dose as fully active sActRII-Fc (Fig. 5B). Thus, the presence of the Fc tag cannot account for the reduced activity of sActRIIB-Fc relative to sActRII-Fc.

We typically observed a doublet with sActRIIB-Fc after electrophoresis, which is consistent with differentially glycosylated forms of the protein and which was not observed for sActRII-Fc (Fig. 5C). Moreover, sActRIIB-Fc is also available from commercial sources in two preparations, one made in insect cells and the other in mammalian cells. Interestingly, the preparation from insect cells is listed at 10-fold more potent than the mammalian product by the vendor despite similar levels of purity, suggesting that differences in carbohydrate could account for the reduced affinity and bioactivity of sActRIIB-Fc produced in mammalian cells relative to sActRII-Fc. Deglycosylation of both sActRII-Fc and sActRIIB-Fc resulted in single bands equivalent in molecular mass to the lower molecular mass band observed for sActRIIB-Fc before deglycosylation and identical to that of sActRII-Fc (Fig. 5C). However, deglycosylation failed to alter the biological activity of sActRII-Fc or sActRIIB-Fc (Fig. 5D), indicating that biological differences between these two receptors are not influenced by glycosylation state.

Activin Binding of sActRII-Fc/sActRIIIB-Fc Complexes—Based on the recent crystal structure solutions for several TGF-β ligands bound to type II receptor ECDs and the increase in affinity of sTβRII-Fc for TGF-β2 in the presence of sTβRI-Fc (18), we hypothesized that the affinity of sActRIIB-Fc for activin might increase to that observed for sActRII-Fc when complexed with ActRIA or ActRIB. We therefore investigated whether sActRIA-Fc or sActRIB-Fc can cooperate with sAct-RII-Fc or sActRIIB-Fc in solution and alter the affinity of the complex. sActRIA-Fc or ActRIB-Fc was combined in solution with either sActRIFc or sActRIIB-Fc and tested for binding to radiolabeled activin A or B (Fig. 6, A and B, respectively). Since we observed that 125I-activin A bound better to sActRIFc than to sActRIIB-Fc (Fig. 6A) when equal amounts of receptor protein were analyzed, the amount of sActRIIB-Fc was increased to compensate for the lower affinity of 125I-activin B (Table I). Binding of 125I-activin A or B to either type I receptor protein alone was negligible. In addition, little additional activin binding was observed when the type II receptors were combined with either type I receptor, indicating that the presence of the type I receptor does not substantially alter the affinity of either type II receptor for activin A or B.

We also explored whether type II/type I combinations could equalize the reduced biological activity of ActRIIB-Fc relative to ActRII-Fc. Consistent with earlier results (Fig. 3), sActRII-Fc (2.44 nM) completely inhibited activin A signaling, whereas at this same dose, sActRIIB had no inhibitory activity (Fig. 6C). In addition, neither sActRIA-Fc nor sActRIB-Fc possessed any inhibitory activity by themselves. Combinations of sActRII-Fc or sActRIIB-Fc with either sActRIA-Fc or sActRIB-Fc did not enhance the biological activity of either sActRII-Fc or sActRIIB-Fc in this assay (Fig. 6C). These results suggest that associations between ECDs of activin type II and type I receptors are not sufficient to alter the binding activity of the type II receptor ECD alone and thus do not account for the difference in binding between sActRII-Fc and sActRIIB-Fc.

Reconstitution of Soluble Inhibin Receptors—Although a high affinity inhibin receptor complex composed of ActRII and TβRIII (betaglycan) was recently identified in transfected cells (17), the possibility that as yet unidentified cell-surface proteins participate in the binding reaction was not excluded. To test whether sActRII-Fc and sTβRIII-Fc could form high affinity inhibin-binding complexes in vitro, radiolabeled inhibin A was incubated with sActRII-Fc and sTβRIII-Fc alone or in combination. sActRII-Fc (0.8 nM) by itself bound 2000 cpm, whereas sTβRIII-Fc (27 nM) bound <1000 cpm (Fig. 7A). However, when 0.8 nM sActRII-Fc was incubated with increasing concentrations of sTβRIII-Fc (0.26–27 nM), 125I-inhibin A binding increased dramatically at sTβRIII-Fc doses above 13 nM or and ~16.5:1 molar ratio with sActRII-Fc. To demonstrate specificity of the complex, we substituted sTβRIII-Fc (0.8 nM) for sActRII-Fc, which did not bind inhibin even when complexed with 27 nM sTβRIII-Fc, indicating that inhibin binding specifically requires both ActRII and TβRIII.

Using a submaximal dose of 0.8 nM sActRII-Fc or sAct-
We have developed a cell-free assay system to quantitatively analyze soluble inhibin and activin receptors. This system affords a number of advantages, including the absence of confounding cell-surface proteins, which can complicate cellular assay systems; the complete control of receptor number and subtype composition; high throughput capability through utilization of the Fc tag and protein A-coated plates; and the ability to create precise stoichiometric combinations containing type I and type II receptors as well as accessory proteins such as TβRII (betaglycan). Using these pure receptor proteins, we have explored the affinity and specificity of each activin type II receptor, ActRII and ActRIIB, for activins A and B as well as inhibins A and B. Consistent with studies using membrane-anchored forms of these receptors (16, 20), we found that both receptors bound activin A with high affinity, that activin A was preferred over activin B, and that inhibin binding to these receptors was at least 10-fold reduced compared with activin. Surprisingly, we were also able to reconstitute the recently identified inhibin receptor (17) in vitro using mixtures of activin type II receptors and a molar excess of stβRIII-Fc. Taken together, these results demonstrate that the purified receptor-Fc fusion proteins can form complexes in vitro that approximate those found naturally on cell membranes and are thus useful tools to explore complex formation and ligand binding.

Current models predict that the functional receptor complex for the TGF-β superfamily is composed of type II and type I receptors that form heterotetramers, suggesting that the signaling complex could be a large multimeric structure containing multiple type II and type I receptors (15). In the human, at least 40 ligands have been identified in the TGF-β superfamily, whereas the repertoire of receptors is much smaller, consisting of seven type I and five type II receptors (reviewed in Ref. 15). Thus, different type II and type I receptors must combine with each other to produce functional receptor complexes with a range of specificities to accommodate this diversity of ligands. Receptor complex specificity can also be altered through accessory proteins such as Cripto, which suppress binding of activin to ActRII and promote nodal binding (33). In this regard, activin type II receptors are a particularly interesting example of promiscuity within this family since they participate in signal transduction for activins, myostatin, nodal, and certain bone morphogenetic proteins (BMPs) through alterations in their subtype composition; high throughput capability through utilization of the Fc tag and protein A-coated plates; and the ability to create precise stoichiometric combinations containing type I and type II receptors as well as accessory proteins such as TβRII (betaglycan). Using these pure receptor proteins, we have explored the affinity and specificity of each activin type II receptor, ActRII and ActRIIB, for activins A and B as well as inhibins A and B. Consistent with studies using membrane-anchored forms of these receptors (16, 20), we found that both receptors bound activin A with high affinity, that activin A was preferred over activin B, and that inhibin binding to these receptors was at least 10-fold reduced compared with activin. Surprisingly, we were also able to reconstitute the recently identified inhibin receptor (17) in vitro using mixtures of activin type II receptors and a molar excess of stβRIII-Fc. Taken together, these results demonstrate that the purified receptor-Fc fusion proteins can form complexes in vitro that approximate those found naturally on cell membranes and are thus useful tools to explore complex formation and ligand binding.

Current models predict that the functional receptor complex for the TGF-β superfamily is composed of type II and type I receptors that form heterotetramers, suggesting that the signaling complex could be a large multimeric structure containing multiple type II and type I receptors (15). In the human, at least 40 ligands have been identified in the TGF-β superfamily, whereas the repertoire of receptors is much smaller, consisting of seven type I and five type II receptors (reviewed in Ref. 15). Thus, different type II and type I receptors must combine with each other to produce functional receptor complexes with a range of specificities to accommodate this diversity of ligands. Receptor complex specificity can also be altered through accessory proteins such as Cripto, which suppress binding of activin to ActRII and promote nodal binding (33). In this regard, activin type II receptors are a particularly interesting example of promiscuity within this family since they participate in signal transduction for activins, myostatin, nodal, and certain bone morphogenetic proteins (BMPs) through alterations in their complex partners (15, 34). Taken together, these results demonstrate that the precise composition of signaling complexes on
the cell surface is a critical determinant of which ligands can transduce biological signals in any particular setting. Since the cell culture systems that are routinely used for these studies often contain a variety of TGF-β superfamily receptors, the precise specificity of each combination of receptors has been difficult to define. The purified soluble receptor proteins, as detailed in this study, represent a valuable tool to clarify precisely which receptors interact in complexes to bind the wide array of ligands in this superfamily.

The ActRII ECD monomer binds activin A with an affinity of 2–7 nM and has been used as an activin antagonist to modulate activin A and B activity in vitro (20, 35). However, modeling studies of TGF-β superfamily receptors suggest that the type I and type II receptors exist as multimers in cells even in the absence of ligand (15). It was recently shown that, when obliged to form trimers through addition of heterologous coiled-coil domains, the affinity of sTβRII and sTβRIII for TGF-β was increased such that biological antagonism could be observed (36). Interestingly, the expression system used for the present studies utilizes a portion of the human Fc chain that contains a free cysteine, resulting in the receptor-Fc fusion proteins being secreted as dimers. This dimerization may explain the 40-fold higher affinity we observed for sActRII-Fc relative to the free ECD (29). In fact, the affinity of sActRII-Fc for activin A was ~3-fold higher than that observed for membrane-anchored ActRII (16, 28), suggesting that the configuration of the ActRII ECD that is adopted when constrained by the Fc tag is favorable for binding. Consistent with this interpretation, the potency of sActRII-Fc in inhibiting activin biological activity actually exceeded the potency of the nearly irreversible activin-neutralizing protein follistatin (37). On the other hand, the affinity of sActRII-Fc for activin B was 60-fold lower than for activin A, suggesting that this receptor can distinguish between the two activins in a biologically meaningful way.

In comparison with sActRIIA-Fc, the affinity of sActRIIB-Fc for activin A was 7-fold lower, whereas that for activin B was actually slightly higher. Thus, the difference in affinity between activins A and B was only 6-fold for sActRIIIB-Fc as compared with the 60-fold difference observed for sActRII-Fc. The reduced affinity of sActRIIB for activin A was mirrored in its biological activity, where ActRIIB-Fc was ~10-fold less active than ActRIIA-Fc. Neither removal of the Fc tag nor deglycosylation altered this difference in biological activity between sActRIIA-Fc and sActRIIB-Fc. However, despite being lower than that of sActRIII-Fc, the affinity of sActRIIB-Fc for activin A was similar to that observed for natural membrane-bound ActRIIB (100–380 pM) (30). It remains unclear, however, to what extent the characteristics of the soluble receptors reflect those of the natural membrane-bound receptors since it has been difficult to characterize individual naturally occurring receptors in cell-culture systems because of the existence of numerous TGF-β superfamily receptors found on typical cells. However, if the characteristics of soluble receptors do reflect those of membrane-bound receptors, our observations might explain, at least in part, the non-overlapping actions of activins A and B observed in mouse genetic knockout studies (38) as well as the different phenotypes observed for the ActRII and ActRIIB knockout mice (39, 40).

Another possible explanation for the different binding characteristics of sActRIIIB-Fc and sActRIIA-Fc is that the latter may require both type II and type I receptors to stabilize the ligand-receptor complex. To test this hypothesis, we examined binding of radiolabeled activins A and B to each type I and type II soluble receptor alone and in combination. Although little activin bound to either activin type I receptor, binding of activin A or B to the type II receptors was not substantially altered by the presence of either type I receptor. Thus, in contrast to TβRII, which shows enhanced binding to TGF-β2 in the presence of TβRI (18), neither sActRIIB-Fc nor sActRIIB-Fc appears to be similarly influenced by sActRIIA-Fc or sActRIIA-Fc and thus does not provide evidence for cooperativity.

When the two activins and the two inhibins were compared as competitors for radiolabeled activin A binding to sActRIIA-Fc, activin A was clearly the preferred ligand. For example, activin A was ~4–5-fold less potent than activin B, even when competing with 125I-activin B. These observations suggest that this difference in binding was not due to effects of the iodination process. Similarly, the two inhibins were approximately equal to each other in activity, but 20–30-fold less active than activin A. This pattern of ligand preference is similar to that reported for membrane-anchored ActRII (16), consistent with the receptor-Fc fusion proteins faithfully reproducing the characteristics of the natural receptor.

After a nearly decade-long search, one high affinity inhibin
receptor was recently identified consisting of ActRII complexed with TgRIII (betaglycan) (17). Although binding was demonstrated in several cell types, including pituitary Att20 cells, the biological activity of this receptor was demonstrated by transfection of these cells with TgRIII cDNA (they already express ActRII), which reconstitutes the ability of inhibin to inhibit activin action (17). More recent studies in cells demonstrated further that inhibin can suppress the activity of BMPs that bind through ActRII as well as the BMP type II receptor (41). However, since these studies utilized cell culture systems to assess inhibin activity, they could not exclude the possibility that unidentified membrane proteins could be participating in this inhibin-binding complex. Our results with purified sActRIIB-Fc or sActRIIB-Fc combined with purified sTgRIII-Fc in a cell-free assay clearly demonstrate that these two receptors are both necessary and sufficient for high affinity inhibin binding. They further suggest that sActRIIB-Fc/sTgRIII-Fc has a slightly higher affinity for inhibin compared with sActRII-Fc/sTgRIII-Fc. Interestingly, the greatest increase in inhibin binding occurred when the ratio of sTgRIII-Fc to sActRII-Fc was >16–33:1. This is consistent with cell transfection studies in which increasing amounts of TgRIII produced increased inhibin binding when ActRII was present (17, 41), suggesting that inhibin-responsive tissues require a large quantity of TgRIII relative to ActRII.

Crystal structure solutions for the BMP-7:ActRIIA complex (19), the activin A:ActRIIB complex (42), and the TGF-β3:TgRII complex (43) suggest different modalities of receptor complex assembly. Specifically, the crystallographic solution of ActRII complexed with BMP-7 and the included modeling of a complex with the type I receptor suggest that activin bridges the type II and type I receptors, which do not interact directly with each other (19). Another model was proposed for the complex of activin A bound to the ActRIIB ECD in a close packed arrange-
ment with the type I receptors, including direct contacts between the type I and type II receptors suggestive of a cooperative mode of interaction (42). In support of this cooperative model, a small increase in affinity of ActRII for activin A was observed when 293 cells were cotransfected with each soluble type II or type I receptor alone or combined with two doses of type I receptor. As expected, little activin binding was observed to either type I receptor alone. In addition, combining sActRII-Fc or sActRIIB-Fc with each type I receptor added little to the activin binding expected from each receptor alone, indicating that the type II-type I receptor complex added little to the overall affinity of ligand binding established by the type II receptor alone. Note that for radiolabeled activin A, the dose of sActRII-Fc and sActRIIB-Fc was constant so that the reduced binding of activin to sActRIIB resulted in overall reduced counts in the complexes relative to sActRII-Fc. Since activin B binding to either receptor was lower compared with activin A, the dose of ActRIIB was increased to 24 nM in B, where radiolabeled activin B was examined. Nevertheless, the overall result was the same: no increase in activin B binding was observed for the type II-type I receptor complexes compared with each receptor alone. To investigate whether activin receptor complexes have increased bioactivity, each receptor alone or mixed in complexes was tested for inhibition of activin-stimulated CAGA reporter activity (C). Although sActRII-Fc was able to completely inhibit activin activity alone or in complex with type I receptors, no activity was observed at 2.44 nM sActRIIB-Fc (this dose had little activity in Fig. 3) alone or when complexed with type I receptors. Thus, the presence of type I receptors does not appear to influence the binding or biological activity of the soluble activin type II receptors. RLU, relative light units.

FIG. 6. Effect of combining soluble activin type II receptors with soluble type I receptors. To determine whether complexes of soluble activin type II and type I receptors result in stabilization of the ligand-receptor complex, radiolabeled activin A (A) or activin B (B) was incubated with each soluble type II or type I receptor alone or combined with two doses of type I receptor. As expected, little activin binding was observed to either type I receptor alone. In addition, combining sActRII-Fc or sActRIIB-Fc with each type I receptor added little to the activin binding expected from each receptor alone, indicating that the type II-type I receptor complex added little to the overall affinity of ligand binding established by the type II receptor alone. Note that for radiolabeled activin A, the dose of sActRII-Fc and sActRIIB-Fc was constant so that the reduced binding of activin to sActRIIB resulted in overall reduced counts in the complexes relative to sActRII-Fc. Since activin B binding to either receptor was lower compared with activin A, the dose of ActRIIB was increased to 24 nM in B, where radiolabeled activin B was examined. Nevertheless, the overall result was the same: no increase in activin B binding was observed for the type II-type I receptor complexes compared with each receptor alone. To investigate whether activin receptor complexes have increased bioactivity, each receptor alone or mixed in complexes was tested for inhibition of activin-stimulated CAGA reporter activity (C). Although sActRII-Fc was able to completely inhibit activin activity alone or in complex with type I receptors, no activity was observed at 2.44 nM sActRIIB-Fc (this dose had little activity in Fig. 3) alone or when complexed with type I receptors. Thus, the presence of type I receptors does not appear to influence the binding or biological activity of the soluble activin type II receptors. RLU, relative light units.
the ligand type II and type I receptors interact in a cooperative manner and that TGF-β/H18528 type I and type II receptors are both essential for high affinity binding of TGF-β/H18528. In addition, these studies support the utility of the receptor-Fc fusion proteins described herein for testing hypotheses of possible cooperativity generated by crystallographic solutions of ligand/receptor complexes.

Our results demonstrate that purified soluble activin receptor-Fc fusion proteins bind activin with high affinity equivalent to or exceeding that of the natural membrane-anchored protein and far exceeding that of the soluble ECD. These reagents allow complete characterization of the affinity and specificity of the binding sites contained within the ECD and are likely to be characteristic of the natural ECD in the absence of other cellular influences. Moreover, these reagents can be combined to test whether different functional complexes can be produced, as seen in this study for the inhibin receptor complex. They are also useful to examine the nature of receptor complex formation predicted from crystallographic studies since our results support a non-cooperative model of interaction suggested by the lack of type I/type II receptor contact observed in the BMP-7/ActRII crystallographic solution (19) but a cooperative mode of association for TGF-β/H18528 T/RII (18, 43).

Finally, the high affinity of the ActRII-Fc protein for activin A and the 60-fold difference compared with activin B, together with its in vitro biological activity exceeding that of follistatin, suggest that this reagent might be a useful antagonist of activin A in vivo.

### TABLE III

**Affinity of inhibins A and B for soluble inhibin receptor complexes**

Complexes of sActRII-Fc or sActRIIB-Fc with sTβRIII-Fc were incubated with radiolabeled inhibin A and increasing concentrations of unlabeled inhibin A or B to generate inhibition curves as depicted in Fig. 7. The ED_{50} values were calculated from the binding curves and potency relative to unlabeled inhibin A competing with iodinated inhibin A. The dissociation constants (K_d) were determined from Scatchard analysis of these curves. Results are the mean ± S.E. of at least three replicate experiments.

| Receptors       | Radioligand | Competitor | ED_{50} (nM) | Relative potency | K_d (nM) ± S.E. |
|-----------------|-------------|------------|--------------|-----------------|-----------------|
| ActRII + TβRIII | Inhibin A   | Inhibin A  | 10.5 ± 1.3   | 1               | 1110 ± 169      |
| ActRII + TβRIII | Inhibin A   | Inhibin B  | 7.2 ± 0.8    | 1.5             | 1270 ± 37       |
| ActRIIB + TβRIII| Inhibin A   | Inhibin A  | 4.1 ± 0.3    | 2.6             | 433 ± 27        |
| ActRIIB + TβRIII| Inhibin A   | Inhibin B  | 6.4 ± 0.8    | 1.6             | 704 ± 59        |

**FIG. 7.** Reconstitution of soluble high affinity inhibin receptors. A, investigation of radiolabeled inhibin A binding to sActRII-Fc, sTβRII (TBR II)-Fc, sTβRIII-Fc, and combinations of these receptors at the doses shown. Whereas 2000 cpm bound to sActRII-Fc alone, only 1000 cpm bound to sTβRIII-Fc alone or to TβRII used as a control (i.e. indicates nonspecific binding). When sActRII-Fc was mixed with increasing amounts of sTβRIII-Fc, substantial increases in inhibin binding were observed at doses above 13 nM. When sTβRII-Fc was substituted for sActRII-Fc, only nonspecific binding was observed, indicating that inhibin binding is specific for activin type II receptors complexed with TβRII. B, binding competition curves and Scatchard analysis for sActRII-Fc-sTβRIII-Fc complexes showing high affinity binding for this soluble complex similar to affinities reported for the membrane-bound complex (17). C, competitive binding curves and Scatchard analysis for sActRIIB-Fc-sTβRII-Fc complexes showing 2-fold higher affinity for this combination than observed for sActRII-Fc-sTβRII-Fc complexes in B. The data shown are representative of three experiments, the means of which are shown in Table III. Inh A, inhibin A; Inh B, inhibin B.
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