Identification of a Binding Site on the Type II Activin Receptor for Activin and Inhibin*

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Type II activin receptors (ActRII and ActRIIB) are single-transmembrane domain serine/threonine kinase receptors that bind activin to initiate the signaling and cellular responses triggered by this hormone. Inhibin also binds type II activin receptors and antagonizes many activin effects. Here we describe alanine scanning mutagenesis of the ActRII extracellular domain. We identify a cluster of three hydrophobic residues (Phe42, Trp60, and Phe83) that, when individually mutated to alanine in the context of the full-length receptor, cause the disruption of activin and inhibin binding to ActRII. Each of the alanine-substituted ActRII mutants retaining activin binding maintains the ability to form cross-linked complexes with activin and supports activin cross-linking to the type I activin receptor ALK4. Unlike wild-type ActRII, the three mutants unable to bind activin do not cause an increase in activin signaling when transiently expressed in a corticotroph cell line. Together, our results implicate these residues in forming a critical binding surface on ActRII required for functional interactions with both activin and inhibin. This first identification of a transforming growth factor-β family member binding site may provide a general basis for characterizing binding sites for other members of the superfamily.

Activins and inhibins (1) belong to the transforming growth factor-β (TGF-β) family of growth and differentiation factors (2) and were originally identified based on their role in regulating reproductive function. Activin was first identified and isolated based on its ability to stimulate the release of follicle-stimulating hormone from gonadotrophs in the anterior pituitary (3), whereas inhibin was identified based on its ability to inhibit this process (4). Activins (~28,000 Da) and inhibins (~32,000 Da) are disulfide-linked dimers of related polypeptides, with activins consisting of two β chains (activin A, βA; activin AB, βA-βB; and activin B, βB-βB) and inhibins possessing an α chain disulfide-linked to a β chain (inhibin A, α-βA; and inhibin B, α-βB) (1). The structure of these factors is determined by several conserved cysteine residues that form disulfide bonds in the tightly folded “cystine-knot” motif shared by TGF-β superfamily members (6). Although inhibins frequently counteract the effects of activins (7), there are cell types in which inhibin is unable to attenuate the activin response (8, 9), making it unlikely that inhibin blocks activin effects exclusively by competing with activin for receptor binding as has been previously suggested (10).

Type II activin receptors (ActRII and the closely related ActRIIB) are single-transmembrane domain serine/threonine kinase receptors that bind activin with high affinity and thereby initiate the cellular responses triggered by activin (11–14). Like TGF-β receptors (15), activin receptors are thought to exist as homodimers, a finding consistent with their proposed role in binding dimeric ligand. Inhibin also binds ActRII and ActRIIB, although with an ~10-fold lower affinity than has been shown for activin (11, 12). It has been postulated that inhibin exerts its effects by binding to its own distinct receptor component(s) in addition to ActRII (8, 16), although such putative inhibin-specific receptors remain to be described. After binding activin, the type II receptor-activin complex interacts with the type I activin receptor ALK4 (13) to form a heteromeric receptor complex. As with other type II TGF-β family member receptors, the type II activin receptors phosphorylate type I receptors on serine and threonine residues in conserved cytoplasmic juxtapamemembrane domains to activate the type I receptor (14). Following activation, the type I receptors phosphorylate and activate Smad proteins, which subsequently associate with the common mediator Smad protein, Smad4, and then migrate to the nucleus, where they initiate transcriptional responses (17, 18).

To characterize the initial steps in the activin signaling cascade, we have focused on identifying the residues in the ECD of ActRII required for activin and inhibin binding. We recently expressed and characterized the soluble ECD of ActRII and showed that it is capable of binding activin and inhibin and that it competitively inhibits the ability of activin to stimulate follicle-stimulating hormone secretion from gonadotrophs, presumably by competing with full-length ActRII on gonadotrophs for activin binding (19). In addition, we recently solved the crystal structure of the ActRII ECD (20) expressed and purified from yeast (21). The ActRII ECD consists of seven disulfide-cross-linked β sheets that form a three-finger toxin-like fold similar to the folds of several cobra cardiotoxins (22). By solving the ECD crystal structure, we were able to identify surface-exposed amino acid residues that may play an important role in protein-protein interactions such as receptor-ligand or receptor-receptor binding interactions (20).
To identify the amino acid residues on ActRII required for activin and inhibin binding, we performed alanine scanning mutagenesis of the receptor ECD. We focused on amino acid residues shown by the crystal structure to be exposed at the surface of the molecule and therefore potential candidates for protein-protein interactions. Residues were also chosen if they were conserved between mouse (11) and Drosophila (23) type II receptors, two evolutionarily divergent proteins that have a similar high affinity for activin A. Using this approach, we have identified three conserved hydrophobic residues (Phe42, Trp69, and Phe35) that, when mutated individually to alanine, yield receptors with disrupted activin A and inhibin A binding. The ECD crystal structure shows that these residues interact with each other to form a hydrophobic surface that is a likely binding interface (20). We present evidence in this report that these three residues together form a site on ActRII required for both activin A and inhibin A binding to ActRII and for a functional activin response in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**NuPAGE gels and molecular weight markers were obtained from Invitrogen (Carlsbad, CA). Primer pairs for mutagenesis and to subclone mutant polymerase chain reaction products were obtained from Pierce. Mouse ActRII (11) and human ALK4 (13) recombinant human activin A and inhibin A were generated using a stable activin-expressing cell line generously provided by Dr. J. Mathen (Genentech, Inc., South San Francisco, CA), and were purified by Wolfgang Fischer (Peptide Biology Laboratory, Salk Institute). 5'-Activin A and 5'-Inhibin A were prepared using the chloramine-T method as described previously (24), with resulting specific activities ranging from 50 to 100 Ci/μg. Anti-Myc monoclonal antibody (9E10) and protein G-agarose were purchased from Calbiochem. Affinity-purified rabbit anti-ActRII polyclonal antibodies (directed against residues 482–494 of mouse ActRII) have been previously described (25). Horseradish peroxidase-linked anti-rabbit Igg and anti-mouse IgG, 3,3',5,5' tetramethylbenzidine (TMB) peroxidase substrate, and chemiluminescent substrate (Supersignal[23]) were obtained from Pierce. The mouse ActRII (11) and human ALK4 (13) clones used in this study were in the pcDNA3 expression vector (Invitrogen, Carlsbad, CA).

**Mutagenesis of Full-length Mouse ActRII—**To incorporate the amino-terminal Myc epitope and to generate mutations in the extracellular domain of full-length mouse ActRII, we utilized an overlapping polymerase chain reaction strategy. We identified a Myc epitope at a site just 5' of the transmembrane domain in the ActRII construct, allowing us to make and to subclone mutant polymerase chain reaction products (~500 base pairs) spanning only the ECD of the receptor. Primers were constructed to incorporate a 5'-EcoRI site and a 3'-AgeI site, and polymerase chain reaction products were gel-purified and digested with both enzymes and then subcloned into AgeI/EcoRI-digested mouse ActRII construct to yield full-length receptor constructs. For each construct, the mutated amino-terminal ECD region was confirmed by DNA sequencing.

**Transfection, Preparation of Membranes and Soluble Extracts, and Detection of Cell-surface Expression of ActRII in Intact HEK 293 Cells—**HEK 293 cells were grown in 5% CO2 to 70% confluence in Dulbecco's modified Eagle's medium (with 10% bovine calf serum, penicillin, streptomycin, and L-glutamine) and then transfected complete Dulbecco's modified Eagle's medium (with 10% bovine calf serum) with 3 μg of either 5'-EcoRI- or 5'-AgeI-digested mouse ActRII plasmid. Transfected cells were incubated at 5% CO2 for 48 h, and then harvested by rinsing each plate with HDB, blocked with 3% bovine serum albumin in HDB for 30 min at room temperature, rinsed with HDB, and incubated for 2 h with 2 μg/ml anti-Myc antibody in 3% bovine serum albumin in HDB, rinsed three times with HDB, and incubated with peroxidase-conjugated anti-mouse IgG in 3% bovine serum albumin in HDB for 1 h at room temperature. Wells were rinsed three times with HDB; 100 μl of TMB peroxidase substrate was added to each well, and plates were incubated at room temperature until a blue color was visible. Reactions were stopped by adding 100 μl of 0.18 M H2SO4 to each well, and peroxidase activity was quantified by measuring the absorbance of the resulting yellow solutions at 450 nm.

**Membrane Binding—**Binding was performed in 96-well 0.2-μm Durapore plates using the vacuum filtration multiscreen assay system (Millipore). Each well contained a total of 200 μl of 50 μl of binding buffer (HDB with 0.1% bovine serum albumin, 5 mM MgSO4, and 1.5 mM CaCl2), 50 μl of unlabeled competitor (activin A or inhibitin A) at various dilutions in binding buffer, 50 μl of 125I-activin A (~100,000 cpm/well) or 125I-inhibitin A (~300,000 cpm/well) diluted in binding buffer, and 50 μl of HEK 293 cell membranes (~5 μg/ml) diluted in binding buffer. Plates were incubated for 1 h at room temperature, vacuum-filtered, washed twice with 50 μl well binding buffer, and allowed to dry, and individual filters were punched out and counted using a γ-counter. Binding data were analyzed using the Prism program.

**Polyacrylamide Gel Electrophoresis, Immunoblotting, and Covalent Cross-linking—**SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions on NuPAGE gels. Electrophoretically blotted nitrocellulose membranes were carried out in a Novex X-cell II apparatus according to the manufacturer’s instructions. Unbound sites were blocked either for 30 min at room temperature or overnight at 4 °C with 5% (w/v) skim milk powder in Tris-buffered saline (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl).

For ActRII detection, blocked membranes were washed three times for 10 min each with Tris-buffered saline containing 0.05% Tween 20 (TBST) and then incubated for 1 h at room temperature with 2 μg/ml anti-Myc antibody (9E10). Membranes were then washed three times for 10 min each with TBST and incubated for 30 min with 2 μg/ml peroxidase-linked anti-mouse IgG. Blots were washed three times for 10 min each with TBST, and reactive bands were visualized using the Pierce Supersignal[23] ECL detection system.

Covariant cross-linking was carried out by incubating ~106 HEK 293 cells resuspended in HDB with ~2 × 106 cpm 125I-activin A in a total of 500 μl of 1 h at room temperature with gentle rocking. Following this incubation, 1 ml of cold HDB was added to each tube, and cells were then pelleted by centrifugation, resuspended in 500 μl of HDB, brought to 0.5 μM disuccinimidyl suberate, and incubated for an additional 30 min. All cross-linking reactions were quenched by adding 1 ml of Tris-buffered saline to each tube; cells were pelleted by centrifugation and aspirated; and cell pellets were solubilized in 1 ml of lysis buffer on ice for 30 min. Triton X-100-insoluble material was removed by centrifugation; 2 μg of anti-Myc antibody was added to each supernatant; tubes were incubated for 16 h at 4 °C; and immune complexes were precipitated by adding 10 μl of 50% protein G-agarose slurry to each tube, incubating for an additional 1 h at 4 °C, and pelleting the resulting immunobilized immune complexes by centrifugation. Each protein G-agarose pellet was washed three times with 1 ml of lysis buffer, boiled for 10 min, eluted in 25 μl of NuPAGE SDS sample buffer (Novex), and resolved via SDS-polyacrylamide gel electrophoresis.

Luciferase Assays in AtT20 Corticotroph Cells—The activin/TGF-β-responsive luciferase reporter plasmid 3TPLux has been previously described (13) and was generously provided by Dr. Joan Massagué. Mouse AtT20 cells were trypsinized and plated at a density of 2.0 × 105 cells/well in 2 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 2 mM glutamine for 24 h prior to transfection. Cells were transfected in complete medium with ~1 μg of 3TPLux plasmid, 0.1 μg of cytomegalovirus-β-galactosidase plasmid, and 0.2 μg of either vector control or 5'-EcoRI or 5'-AgeI-digested mouse ActRII plasmid. Cells were then harvested in optimized conditions using Superfect transfection reagent (QIAGEN, Hilden, Germany). After incubating for 2.5 h with transfection reagent and DNA, cells were washed and allowed to recover for 5 h in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum and 2 mM glutamine. The cells were treated with activin A for ~16 h and then harvested in solubilization buffer (1% Triton X-100, 25 mM glycyglycine...
FIG. 1. Amino acid residues selected for alanine scanning mutagenesis are surface-exposed and conserved between mouse and Drosophila type II receptors. A, crystal structure of the ECD of ActRII (residues 6–102) represented as a space-filling model showing the concave (left) and convex (right) faces. Hydrophobic surface residues are shaded black, and charged residues are shaded gray. The residues selected for mutation to alanine are indicated, with the three hydrophobic residues identified as required for activin A and inhibin A binding depicted in white lettering. B, sequence alignment of three activin-binding receptor extracellular domains: mouse ActRII (mActRII), mouse ActRIIB (mActRIIB), and the Drosophila type II dpp receptor (AtrII). The positions of the putative signal peptide, the introduced Myc epitope tag, the transmembrane domain, and the conserved surface-exposed residues in mouse ActRII selected for mutation to alanine (boxed) are indicated. The numbering shown corresponds to mouse ActRII.

RESULTS

Selection of Amino Acid Residues on ActRII for Mutagenesis—Fig. 1A shows a space-filling diagram of the ActRII ECD structure (residues 6–102) (20) illustrating both the concave (left) and convex (right) faces of the molecule and indicating the locations of the residues we selected for individual mutation to alanine. Most of the residues selected are conserved between mouse (mouse ActRII and ActRIIB) and Drosophila (activin receptor type II (AtrII)) type II receptors, each of which binds activin with high affinity (Fig. 1B). We predicted that by individually mutating conserved residues to alanine, we would be able to identify amino acids that are important for activin, inhibin, and possibly ALK4 binding while minimizing the structural changes caused by mutation.

Expression and Activin Binding of Myc Epitope-tagged ActRII in HEK 293 Cells—To facilitate detection of ActRII expressed in HEK 293 cells, we used polymerase chain reaction to introduce a Myc epitope tag (EQKLISEEDL) at the extreme amino terminus immediately following the putative signal peptide. We have previously shown by peptide sequencing that the amino termini of the majority of soluble recombinant ActRII ECDs expressed in insect cells begin with the sequence AILG, but that a small fraction of the N-terminal sequences start with ECDs expressed in insect cells begin with the sequence AILG, but that a small fraction of the N-terminal sequences start with the amino terminus immediately following the putative signal peptide, the introduced Myc epitope tag, the transmembrane domain, and the conserved surface-exposed residues in mouse ActRII selected for mutation to alanine (boxed) are indicated. The numbering shown corresponds to mouse ActRII.

To demonstrate that the Myc-tagged ActRII constructs were expressed in HEK 293 cells, we initially performed Western blot experiments. Fig. 2A shows that ActRII, with or without the amino-terminal Myc tag, was expressed in HEK 293 cells. An antibody directed against ActRII recognized an ~65-kDa protein expressed in cells transfected with ActRII or ActRII-Myc, but not vector alone (Fig. 2A, left), whereas an anti-Myc monoclonal antibody detected ActRII-Myc, but not ActRII (right). To demonstrate that the Myc-tagged ActRII constructs were expressed at the cell surface, we developed an intact cell enzyme-linked immunosorbent assay-based assay as described below (see Fig. 4B). These results demonstrate that the Myc epitope is present on the ActRII-Myc construct, that both the untagged and Myc-tagged receptors are expressed in this system at similar levels, and that the receptors are expressed at the cell surface.

To measure activin A binding to both ActRII and ActRII-Myc, we expressed each in HEK 293 cells, prepared cell membranes, and performed competition binding with 125I-activin A. Fig. 2B shows the resulting competition curves and demonstrates that the Myc epitope had little effect on the ability of ActRII to bind activin A. Although we could detect very low levels of displaceable activin A and inhibin A binding in HEK 293 cells transfected with empty vector, this level of binding was negligible in comparison to the binding seen in cells transfected with ActRII (data not shown).

Activin Binding Properties of ActRII ECD Mutants—To identify amino acid residues on ActRII required for binding activin, we expressed a series of ActRII point mutants in which individual selected residues in the ECD were changed to alanine. Most of the residues selected are conserved between mouse (mouse ActRII and ActRIIB) and Drosophila (activin receptor type II (AtrII)) type II receptors, each of which binds activin with high affinity (Fig. 1B). We predicted that by individually mutating conserved residues to alanine, we would be able to identify amino acids that are important for activin, inhibin, and possibly ALK4 binding while minimizing the structural changes caused by mutation.

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To demonstrate that the Myc-tagged ActRII constructs were expressed in HEK 293 cells, we initially performed Western
and mutant ActRII-Myc constructs. As indicated in Table I, we identified three residues in the ECD (Phe42, Trp60, and Phe83) that yielded no detectable binding. We were unable to detect the G58A mutant following transfection into HEK 293 cells by either Western blotting or activin A binding, indicating that this mutant was not expressed. All of the other alanine mutants tested had IC50 values that were comparable to that measured for wild-type ActRII (0.11 (0.02–0.66) nM), with values ranging from 0.1 nM for D77A to 0.7 nM for K37A (Table I). Together, these data strongly suggest that Phe42, Trp60, and Phe83 form a binding site for activin A on ActRII and that the other surface-exposed residues examined are not required for activin A binding.

### Table I

| ActRII-Myc IC50 (nM) |
|----------------------|
| WT                   |
| F42A                 |
| W60A                 |
| F83A                 |
| G58A                 |
| V81A                 |
| V28A                 |
| F13A                 |
| F14A                 |
| I52A                 |
| I54A                 |
| D34A                 |
| D36A                 |
| D62A                 |
| E29A                 |
| D77A                 |
| E10A                 |
| D71A                 |
| K37A                 |
| K56A                 |

* WT, wild-type.

Three Interacting Hydrophobic Residues in the ActRII ECD Are Required for Binding Activin A and Inhibin A—Because activin is a β-β dimer and inhibin is an α-β dimer and also because activin and inhibin can compete for binding to ActRII, it is likely that the β subunit mediates the interaction between each ligand and ActRII and that both activin and inhibin bind to the same or overlapping sites on the receptor. To test whether Phe42, Trp60, and Phe83 are required for activin A and inhibin A binding, we performed competition binding experiments using 125I-activin A and 125I-inhibin A in the presence of 25 nM unlabeled activin A or inhibin A, respectively. Fig. 3A shows that 125I-activin A bound wild-type ActRII and that this binding was blocked in the presence of 25 nM unlabeled activin A. Membranes from cells expressing the F42A, W60A, and F83A mutants displayed very low levels of activin A binding that were not displaced by unlabeled activin A (Fig. 3A). A similar result was obtained with 125I-inhibin A, with displacable binding observed with wild-type ActRII, but not with F42A, W60A, or F83A. The percent of bound ligand relative to total ligand used in these experiments was typically severalfold higher for activin than for inhibin, likely reflecting the higher affinity of activin than inhibin for ActRII. In summary, the three ActRII ECD residues Phe42, Trp60, and Phe83 are each required for both activin A and inhibin A to bind ActRII.

Because these three mutants did not have detectable activin A or inhibin A binding, we performed additional experiments to verify their expression. Fig. 3A shows, by Western blot analysis using the anti-Myc antibody, that the F42A, W60A, and F83A mutants were expressed at similar levels and migrated at similar positions following SDS-polyacrylamide gel electrophoresis relative to the wild-type receptor. In some cases, ActRII migrated as a doublet, most likely reflecting variable levels of glycosylation of the ECD (Fig. 4A). To further demonstrate that each of these mutant receptors was expressed at the cell surface, we developed and employed an enzyme-linked immunosorbent assay-based assay that allowed detection of the Myc epitope on the surface of intact cells using an anti-Myc monoclonal antibody. This assay allowed us to demonstrate that each of the three mutant receptors deficient in activin A and inhibin A binding displayed its amino-terminal Myc epitope at the surface of the cell (Fig. 4B). The levels of cell-surface expression measured for these three mutants varied, and only the W60A mutant was consistently measured at levels comparable to wild-type levels. However, both the F42A and F83A were consistently expressed at levels higher than those with vector alone, and each was expressed at levels as high as those for mutants for which activin A binding was clearly detected (data not shown).

Covariant Cross-linking of Wild-type and Mutant ActRII Constructs to Activin and ALK4—To further characterize the ECD mutants retaining activin A binding, we coexpressed them in
HEK 293 cells along with the type I activin receptor ALK4 (ActRIB) and performed covalent cross-linking experiments. Activin A-cross-linked bands were not detected in cells transfected with vector alone, and activin A did not bind ALK4 in the absence of ActRII (Fig. 5A), as has been previously reported (8, 13, 14). However, when ActRII was expressed alone, the ActRII-activin A-cross-linked band was evident (Fig. 5A), and when ActRII and ALK4 were coexpressed, activin A was cross-linked to both ActRII (upper band) and ALK4 (lower band), in agreement with previous findings (8, 13, 14). Fig. 5B shows that the ActRII mutants tested in this experiment behaved essentially like the wild-type receptor, with the exception of D62A. The D62A mutant was apparently partially degraded as indicated by the shifted pattern of bands in the cross-linking gel (Fig. 5B), and it was unclear whether this mutant supported activin A cross-linking to ALK4. Western blot analysis further demonstrated that the D62A protein was present as two digested Myc-epitope-containing fragments of ~50 and 30 kDa, respectively (data not shown). In summary, although it is possible that there are ActRII ECD residues specifically involved in the interaction between ActRII and ALK4, none of the residues we selected for this study appear to be required in this process.

**Mutation of Amino Acids Required for Activin A Binding Blocks the Ability of ActRII to Mediate Activin Signaling in Intact Corticotroph Cells**—We tested the relative abilities of wild-type and mutant ActRII constructs to mediate an increase in the induction of the activin/TGF-β-responsive luciferase reporter construct 3TPLux in AtT20 corticotroph cells. This cell line has previously been shown to be activin-responsive, with activin causing a reduction in both pro-opiomelanocortin mRNA levels and ACTH secretion (9). As shown in Fig. 6, activin A induced 3TPLux reporter activity when this plasmid was transiently transfected into AtT20 cells. Activin A-induced 3TPLux activation was further enhanced when wild-type ActRII was cotransfected (Fig. 6). However, receptors with alanine substitutions at any one of the three residues identified to be required for high affinity activin A binding (Phe42, Trp60, or Phe83) did not support this increase in activin A-induced luciferase activity (Fig. 6). By contrast, the K56A mutation, which is adjacent to the Phe42-Trp60-Phe83 surface (see Fig. 1A) and binds activin A with an IC50 that is not significantly different from that for the wild-type receptor (see Table I), behaved like wild-type ActRII with respect to its ability to mediate 3TPLux induction by activin A (Fig. 6). Together, these results provide further evidence in intact cells demonstrating the importance of the Phe42-Trp60-Phe83 hydrophobic cluster in mediating ActRII-activin binding and activin signaling.

**DISCUSSION**

The activin signaling pathway plays a critical role in the regulation of a wide array of diverse biological processes, including hormone release, cell proliferation, differentiation, and pattern formation during embryogenesis (1, 27). Understand-
Fig. 5. Covalent cross-linking of $^{125}$I-activin A to wild-type and mutant ActRII constructs and ALK4. A, HEK 293 cells were transfected with empty vector (pcDNA3), wild-type ActRII-Myc, ALK4, or both ActRII-Myc and ALK4 and cross-linked to $^{125}$I-activin A as described under "Experimental Procedures" with cross-linked complexes isolated by immunoprecipitation using an anti-Myc antibody; resolved by SDS-polyacrylamide gel electrophoresis; and visualized by autoradiography. B, HEK 293 cells were transfected with Myc-tagged ActRII mutants and ALK4 and treated as described for A.

membrane binding assay that to our knowledge has not been used previously for measuring activin or inhibin binding. We show that the wild-type receptor expressed in HEK 293 cells binds activin A with high affinity ($IC_{50} = 0.1 \text{nM}$) and that the addition of an amino-terminal Myc epitope tag to the receptor does not affect its ability to bind activin A. After screening our panel of mutant receptors, we identified three mutants (F42A, W60A, and F83A) with no detectable activin A or inhibin A binding. These mutants were shown to be expressed as determined by Western blot experiments and were targeted to the cell surface based on detection of their respective amino-terminal Myc epitopes on intact cells. Together, these results indicate that these three mutants are available at the cell surface to bind ligand, but are unable to do so presumably because the activin/inhibin-binding site is disrupted.

A recent study found that mutation of lysine 102 on activin A to alanine or glutamate blocks the ability of the resulting activin A mutant to bind ActRII and signal (32). Based on our findings, it is unclear which residue(s) on ActRII might interact with this lysine. If Lys$^{102}$ on activin A forms specific binding contacts with ActRII, it might be predicted to form an ionic interaction with a negatively charged acidic residue on the ActRII ECD. However, we individually mutated each of the ECD acidic residues (Asp and Glu) that are conserved between the mouse and Drosophila activin-binding type II receptors, and none of these alanine substitutions significantly affected activin A binding. It is possible that two or more acidic residues in the ActRII ECD can interact with Lys$^{102}$ to form a salt bridge and that when one is mutated, the other can compensate to prevent a significant decrease in affinity for activin A. It is also possible, however, that the Lys$^{102}$ mutation prevents ActRII-activin A binding by causing a localized disruption in activin A folding, but that this residue does not directly mediate the high affinity binding contact with ActRII.

In addition to characterizing activin A binding to mutant ActRII proteins, we also examined the ability of these mutants to interact with the type I activin receptor ALK4. It has been shown that a truncated ActRII mutant lacking its cytoplasmic domain retains its ability to associate with ALK4 following
activin A binding and cross-linking (33). We therefore hypothesized that independent binding sites on ActRII for activin A and ALK4 may exist and that we might be able to identify ECD mutants that bind activin A, but that do not interact with ALK4. After testing our mutants in HEK 293 cells coexpressing ALK4, we were unable to clearly identify an alanine point mutant that bound activin A, but that did not interact with ALK4. It is possible that other surface-exposed ECD residues that we did not mutate are required for specific ActRII-ALK4 interactions or that the transmembrane domain and/or the juxtamembrane cytoplasmic region of ActRII is required for this binding. Alternatively, the activin A ligand itself may independently provide the basis for interactions between ActRII and ALK4, in which case, we would not expect to find ECD mutations in which activin A binding is retained, but ALK4 binding is lost.

We further tested the functional consequences of disrupting the Phe42-Trp60-Phe83 hydrophobic binding surface on ActRII. We first demonstrated that when transfected into corticotroph AtT20 cells, wild-type ActRII increases the level of activin A secretion or that the corresponding residues in the activin-binding receptors ActRIIB and AtriII, further suggesting an important role for these residues in binding activin. The corresponding residues in the type II TGF-β receptor are the less well conserved Val, His, and Met, respectively (20), consistent with the fact that this receptor does not bind activin A. However the type II bone morphogenetic protein (BMP) receptor (34–36), which also does not bind activin A, is highly similar to mouse ActRII at these positions and possesses the residues Tyr, Trp, and Phe, respectively (20). Therefore, although the three residues identified on ActRII are required for ActRII to bind activin A, they are apparently not sufficient for activin A binding in the context of the related type II BMP receptor. Future studies will now be needed to determine whether additional binding determinants on ActRII are required for activin A and/or inhibit A binding and to identify the putative binding site for activin on the type II receptor ALK4. It will also be of interest to establish whether other TGF-β family member receptors possess similar hydrophobic binding surfaces required for binding their respective ligands.

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Identification of a Binding Site on the Type II Activin Receptor for Activin and Inhibin

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