An Activin Mutant with Disrupted ALK4 Binding Blocks Signaling via Type II Receptors*

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Activins control many physiologic and pathophysiologic processes in multiple tissues and, like other TGF-β superfamily members, signal via type II (ActRII/IIB) and type I (ALK4) receptor serine kinases. ActRII/IIB are promiscuous receptors known to bind at least a dozen TGF-β superfamily ligands including activins, myostatin, several BMPs, and nodal. Here we utilize a new screening procedure to rapidly identify activin-A mutants with loss of signaling activity. Our goal was to identify activin-A mutants able to bind ActRII but unable to bind ALK4 and which would be, therefore, candidate type II activin receptor antagonists. Using the structure of BMP-2 bound to its type I receptor (ALK3) as a guide, we introduced mutations in the context of the inhibin βA cDNA and assessed the signaling activity of the resulting mutant proteins. We identified several mutants in the finger (M91E, I105E, M108A) and wrist (activin A/activin C chimera, S60P, I63P) regions of activin-A with reduced signaling activity. Of these the M108A mutant displayed the lowest signaling activity while retaining wild-type-like affinity for ActRII. Unlike wild-type activin-A, the M108A mutant was unable to form a cross-linked complex with ALK4 in the presence of ActRII indicating that its ability to bind ALK4 was disrupted. This data suggested that the M108A mutant might be capable of modulating signaling of activin and related ligands. Indeed, the M108A mutant antagonized activin-A and myostatin, but not TGF-β, signaling in 293T cells, indicating it may be generally capable of blocking ligands that signal via ActRII/IIB.

The transforming growth factor β (TGF-β)1 superfamily comprises 42 members in human (1) that control cell proliferation, cell death, metabolism, homeostasis, differentiation, immune responses, wound repair, endothocrine function, and many other physiologic processes (2–6). Members of this superfamily include the TGF-β (2), activin (5), bone morphogenetic protein (BMP)/growth, and differentiation factor (GDF) (4), and nodal-related families (6). Disruption or dysregulated activity of TGF-β superfamily members is associated with multiple pathologic states resulting from processes including aberrant cellular differentiation, proliferation, and/or metabolism (7, 8). TGF-β superfamily members share a distinct structural framework known as the cystine knot scaffold (9, 10). Activins adopt this prototypical disulfide-linked dimeric structure and consist of two β-chains. Although there are four activin β-subunit genes (βA, βB, βC, and βE) in human and an extensive array of possible β-β dimers, only βA-βA (activin-A), βA-βB (activin-AB), and βB-βB (activin-B) have been isolated as biologically active dimeric proteins (5, 11).

Activins, and other TGF-β superfamily members, exert their biological effects by interacting with two types of cell surface transmembrane receptors (type I and type II receptors) with intrinsic serine/threonine kinase activities (12). The receptor activation mechanism for activin involves initial binding to its type II receptor (ActRII/IIB), which leads to the recruitment, phosphorylation, and activation of its type I receptor (ALK4) followed by activation of intracellular signaling (13, 14). However, the specific amino acid residues of activin-A involved in the underlying intermolecular interactions within the activin/ActRII-ECD/ALK4-ECD complex are only now, based on relevant structural and functional data, being deduced.

Activin and related TGFβ ligands have a single disulfide bond that covalently connects the two chains of the dimer. Based on the crystal structure of related ligands (15) and of activin bound to ActRIIB (16), and with reference to the BMP7-ActRII structure (17), it is predicted that within each activin monomer two pairs of anti-parallel β-strands form first a short and then a long “finger” that stretch out like wings from the cystine-knot core of the dimer. The characteristic curvature of the fingers creates concave and convex surfaces on the butterfly-shaped ligand. At the base of the wing-like fingers, each monomer has an α-helix, which together with the pre-helix loop and the inner concave surface of the fingers of the other monomer, form the “wrist” region. In the crystal structure of the ActRII-ECD bound to BMP7 (17), as well as that of activin bound to ActRIIB (16), the activin type II receptor ECDs make contact with the convex outer face or “knuckle” of the finger region of each ligand. The activin-A:ActRIIIB interface involves hydrophobic (Ile30, Ala31, Pro32, Pro88, Tyr94, and Ile100), and ionic/polar (Arg87, Ser90, Lys102, Glu111) residues on activin-A and overlaps the interface determined in the BMP7:ActRII structure (16, 17).

Less is known about the activin-A:ALK4 interface. Based on the crystal structure of BMP2 in complex with ALK3 (18), it has been predicted that all type I receptors bind to the wrist epitope of their respective ligands, irrespective of whether they...
do so with high or low affinity. Consistent with this model, an allosteric conformational change was observed in the "wrist" region of BMP7 following binding to the ActRII-ECD, and this may allow for the cooperative type II type II receptor assembly induced by TGF-β superfamily members (17). To test whether residues in the wrist region of activin-A are important for binding to ALK4, we have subjected activin-A to both alanine and homology scanning mutagenesis. Using these approaches we have shown that substantial mutations in the α-helix region are required to have any significant effects on activin-A activity. This is in contrast to the importance of the corresponding region in TGF-β binding to ALK5. Of greater importance for activin biological activity and binding to ALK4 were residues on the concave face of finger 2. Mutation of these residues generated activin mutants with disrupted ALK4 binding, dramatically reduced signaling and antagonist/partial agonist activity.

EXPERIMENTAL PROCEDURES

Materials—NuPAGE gels and molecular mass markers were purchased from Invitrogen (San Diego, CA). Recombinant human activin-A was generated using a stable activin-expressing cell line provided by Dr. J. Mather (Genentech, Inc., South San Francisco, CA). 125I-activin-A was prepared using the chloramine T method as previously described (16). Hesperidin-peroxidase-linked anti-mouse IgG, TMB substrate, chemiluminescent substrate (Supersignal™), and the BCA protein assay kit were obtained from Pierce. The activin-A constructs used in this study were in the pcDNA3 expression vector (Invitrogen).

Mutagenesis of Activin-A—To incorporate mutations in the mature region of the full-length rat inhibin βa cDNA, we utilized an overlap-PCR strategy. First, we introduced a unique NheI site just 5′ of the mature region in the inhibin βa construct, allowing us to make and subclone mutant PCR products (−600 bp) spanning only the mature region. Primers were constructed to incorporate a 5′ NheI site and a 3′ XhoI site for subcloning back into the full-length construct. Gel-purified PCR products were digested with NheI and XhoI and then subcloned into NheI/XhoI-digested inhibin βa vector. For each construct, the mutated N-terminal mature region was confirmed by DNA sequencing.

Measurement of Recombinant Activin-A Expressed In and Secreted from HEK293T Cells—293T cells were grown in 5% CO2 to 40% confluence on poly-D-lysine-coated plates in complete Dulbecco’s modified Eagle’s medium (with 10% bovine calf serum, penicillin, streptomycin, and t-glutamine). Cells were transfected with wild-type activin-A cDNA containing the N-terminal mature region of inhibin βa using the PEI transfection reagent. Briefly, 24 μg of activin mutant DNA was diluted to 1.2 ml with serum-free Dulbecco’s modified Eagle’s medium. PEI (10 mg/ml stock) was diluted 1 ml was added to the DNA solution, vortexed, and total DNA was kept constant using pcDNA3.0. After 4 h 293T cells were incubated with 125I-activin-A or 125I-M1018A (10⁵ cpm/well) for 4 h at room temperature in binding buffer (20) with gentle rocking. Cells were washed in HDB, resuspended at 0.5 mM disuccinylsuberate (DSS) in HDB and incubated 30 min on ice. Cross-linking reactions were quenched with TBS (50 mM Tri-HCl, pH 7.5, 150 mM NaCl) and cells were solubilized in lysis buffer (TBS containing 1% Nonidet P-40, 0.5% deoxycholate and 2 mM EDTA) and subjected to immunoprecipitation using anti-Myc or anti-ALK4 antibodies as indicated. Immune complexes were analyzed by SDS-PAGE and autoradiography.

Antagonism Assay in HEK293T Cells—The activin/TGF-β-responsive luciferase reporter plasmid A5-Lux (21) and the FAST-2 transcription factor (21) were used as described (22). 293T cells were plated on poly-l-lysine-coated 24-well plates at a density of 150,000 cells per well. Approximately 24 h later, each well was transfected with 500 ng of DNA: empty vector (pcDNA3; 400 ng), FAST-2 (50 ng), A3-lux (25 ng) and cytomegalovirus (CMV)-β-galactosidase (β-GAL) (25 ng). Transfection was performed under optimized conditions using Perfectin transfection reagent (Gene Therapy Systems). 16 h post-transfection, cells were pretreated with activin-A mutants for 2 h prior to treatment with activin-A, myostatin or TGF-β for 16 h. Cells were harvested in solubilization buffer (1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol), and luciferase reporter activity was measured and normalized relative to β-GAL activities.

FSH Assay in the Lf32 Gonadotrope Cell Line—Lf32 cells were plated in 24-well plates at a density of 1.5 × 10⁵ cells/well. Before initiating experiments, the cells were allowed to recover for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum. The cells were washed three times with the same medium and treated for 72 h as indicated. FSH was quantified by radioimmunoassay with reagents from the National Hormone and Pituitary Program of NIDDK, as previously described (23).

RESULTS

Synthesis and Secretion of Wild-type and Mutant Activin-A by 293T Cells—Synthesis of activin-A was first analyzed in 293T cells using a transient transfection approach. Fig. 1A shows that conditioned medium from 293T cells transfected with the inhibin βa cDNA contains mature, dimeric activin-A protein as detected by Western blot analysis. The concentration of activin-A in the conditioned media of transiently transfected 293T cells after 48 h was generally 5–10 nM based on densitometric analysis of autoradiographs. All the activin mutants described in this study were expressed at levels comparable to wild-type activin-A (data not shown). In order to rapidly screen numerous activin-A mutants for biological activity we utilized a luciferase-based assay system in HEK293T cells (22). 293T...
A representative experiment is shown. This experiment was repeated four times with similar results and a untreated cells is shown with "tivin-A and assayed for luciferase activity as described under mental Procedures. 

The expression and activity of activin-A following transfection of 293T cells. A, conditioned media from 293T cells transfected with the inhibin βA cDNA was subjected to Western blot analysis using an anti-activin βA antibody as described under “Experimental Procedures.” Purified activin-A (1, 5, and 25 ng) was analyzed in the same manner and used to estimate the concentration of activin-A in the conditioned media. B, 293T cells transfected in triplicate with A3-Lux, CMV-β-galactosidase, and FAST2, were incubated in the presence of increasing concentrations of activin-A conditioned media or 2 nM activin-A and assayed for luciferase activity as described under “Experimental Procedures.” Fold induction of activin-A treated cells relative to untreated cells is shown with error bars indicating S.D. from the mean. This experiment was repeated four times with similar results and a representative experiment is shown.

Cells had previously been shown to express all of the components of the activin signal transduction system except for the transcription factor FAST2 (22). Without FAST2, 293T cells do not respond to activin. By co-transfecting 293T cells with the A3-luciferase reporter construct (A3-lux), and FAST2, we generated a system that was exquisitively sensitive to activin stimulation. When conditioned medium from 293T cells transfected with the inhibin βA cDNA was used to treat cells transfected with A3-lux and FAST2 there was a dose-dependent induction of luciferase expression (Fig. 1B). Maximal stimulation was obtained with conditioned medium diluted 1:2 and was comparable to that seen with a maximal dose of activin-A (2 nM, Fig. 1B). These results indicate that 293T cells transfected with the inhibin βA cDNA secrete functional activin-A. We have also demonstrated that this assay can be carried out on cells in which inhibit βA, FAST2, A3-lux, and CMV-β-galactosidase are transfected into the same cells (data not shown). In this case, induction of luciferase is the result of activin-A being secreted and then acting in an autocrine/paracrine manner on the cells that secreted it. These assay systems provided us with a rapid indication of the functional status of individual activin-A mutants.

Selection of Amino Acid Residues on Activin-A for Mutagenesis—We showed that 293T cells secrete functional, dimeric activin-A when transfected with the inhibin βA cDNA (Fig. 1), and we proceeded to introduce mutations within the mature region of the inhibin βA construct with the goal of identifying activin-A mutants able to bind ActRII but unable to bind ALK4 and, therefore, candidate type II activin receptor antagonists. Kirsch et al. (18) identified a large epitope for high affinity ALK3 binding spanning the interface of the BMP-2 dimer (Fig. 2), and we have mutated most of the residues on activin-A individually or in combination which correspond to these BMP-2 residues. In addition, we have mutated activin-A residues in the broader region implicated by the BMP-2/ALK3 complex structure focusing, in particular, on the wrist epitope of activin-A, which comprises residues in the pre-helix loop and the α-helix of one monomer as well as residues in the finger region of the second monomer (Fig. 2). Initially, residues were substituted by alanine and then subsequently charged residues were introduced as these were predicted to be more disruptive. In order to disrupt the organization of the α-helix, residues in this region were also substituted for proline. Finally, we generated activin-A/activin-C chimeras in which the pre-helix loop or α-helix of activin A was replaced with the corresponding regions of the biologically inactive activin-C.

Functional Properties of Activin-A Mutants—We tested the relative abilities of wild-type and mutant activin-A constructs to induce A3-lux in 293T cells. For the initial screen, each mutant was quantitated by densitometric analysis following Western blot and then diluted to 2 nM (data not shown). As shown in Table I, most mutants with changes within the pre-helix loop and α-helix had little effect on activin-A activity. Only substantial mutations, such as exchanging the entire α-helix of activin-A for that of the biologically inactive activin-C analog (A/C-2 mutant), or inserting proline residues at certain points within the α-helix (S60P, I63P), had significant effects on activin-A activity. This is in contrast to the importance of the corresponding residues of BMP2 for binding to ALK3 and highlights important differences in the way activin and BMP2 interact with their type I receptors. Of greater importance for activin activity were residues in finger 2 of the activin-A ligand. In particular, mutation of residues Ser90, Met91, Leu92, Lys102, Ile105, and Met108 had significant effects on activin-A activity (Table I). Residues Ser90, Leu92, and Lys102 have previously been shown to be involved in activin-A binding to ActRII and were included as controls in this assay (17, 24). Residues Met91, Ile105, and Met108 form a pocket on the surface of activin-A and, based on the BMP-2/ALK3 structure, are implicated in type I receptor binding.

Competition Binding Studies of Activin-A Analogues—The low biological activity of some of the activin-A mutants could have been because of either disrupted ActRII or ALK4 binding. To determine whether the identified mutants retained the ability to bind ActRII we performed competition binding assays in which the mutants were assessed for their ability to displace 125I-activin-A from 293T cells transfected with ActRII. The EC50 of activin-A for ActRII in these studies was determined to be ~150 pM. As is shown in Fig. 3A, the three activin-A α-helix mutants with disrupted activity (A/C-2, S60P, I63P) each retained the ability to bind ActRII, as illustrated by their wild-type-like ability to compete for 125I-activin-A binding to 293T cells. In contrast, of the five activin-A mutants tested...
only M108A retained affinity for ActRII comparable to that of wild-type activin-A (Fig. 3B). Mutants I105E and M108E had a 2-fold lower affinity for ActRII than did wild-type activin (~400

**TABLE I**

| Mutant        | Epitope       | Activin activity |
|---------------|---------------|------------------|
| Activin A     |               | 100              |
| W25A          | Finger 1      | 73               |
| W28A          | Finger 1      | ND               |
| A31Q          | Finger 1      | 84               |
| Y35A          | Finger 1      | 101              |
| P45A/S46A/H47A/I48A | Pre-helix | 91               |
| A49G          | Pre-helix     | 82               |
| G50A/T51A     | Pre-helix     | 83               |
| S52A/G53A/S54A | Pre-helix  | 99               |
| S55A/L56A     | Pre-helix     | 101              |
| S57A          | Pre-helix     | 101              |
| F58A/H59A     | Pre-helix     | 126              |
| S60A/T61A     | a-Helix       | 97               |
| V62A/I63A     | a-Helix       | 83               |
| H65A/M68A     | a-Helix       | 82               |
| R69A/G70A/H71A| a-Helix       | 82               |
| S72A/P73A/F74A/A75G | Pre-helix | 104              |
| N76A/L77A/K78A/S79A | a-Helix  | 82               |
| A/C-1 (S46L/T51M/S52P/S54I/S55A/L56A) | Pre-helix | 104 |
| A/C-2 (S60T/T61A/I63L/H65L/R67K) | Pre-helix | 17 |
| M68A/R69N/G70T/H71A/S72A | A/C-2.1 (S60T/T61A/I63L) | a-Helix | 81 |
| A/C-2.2 (H63L/H65L/R67K) | a-Helix | 107 |
| A/C-2.3 (Y66L/R67K/M68A/R69N/G70T) | a-Helix | 87 |
| A/C-2.4 (M68A/R69N/G70T/H71A/S72A) | A/C-2.2.1 (S60T/T61A/I63L) | a-Helix | 81 |
| S57P          | Pre-helix     | 83               |
| H59P          | Pre-helix     | 85               |
| S60P          | a-Helix       | 17               |
| T61P          | a-Helix       | 78               |
| V62P          | a-Helix       | 64               |
| I63P          | a-Helix       | 19               |
| M91A          | Finger 2      | 74               |
| M91E          | Finger 2      | 3                |
| I105A         | Finger 2      | 86               |
| I105E         | Finger 2      | 13               |
| M108A         | Finger 2      | 8                |
| M108E         | Finger 2      | 3                |
| S90A          | Finger 2      | 59               |
| L92A          | Finger 2      | 35               |
| K102E         | Finger 2      | 18               |

**Fig. 2. Sequence alignment of activin-A and BMP-2.** BMP2 residues implicated in ALK3 binding are boxed and shaded gray. Activin-A residues selected for mutagenesis are shaded black, and the residues they were substituted for are indicated. Residues in activin-A known to be involved in binding ActRII, and included in this study as controls, are boxed. The location of secondary structure elements such as β-sheets (indicated by arrows), and the a-helix was adapted from Scheufler et al. (39).

**Fig. 3. Competition binding displacement curves for activin-A analogs.** HEK293T cells were transfected with ActRII and 24–48 h later were subjected to competition binding as described under "Experimental Procedures." Displacement curves for activin-A analogs with mutations in the a-helix region (A), including the A/C-2 (closed circles), S60P (open triangles), and I63P (open squares) mutants, and the finger region (Fig. 3B), including the M91E (closed circles), K102E (open squares), I105E (open circles), and M108E (open diamonds) mutants, are shown. The displacement curve generated in the presence of unlabeled activin-A is shown for comparison (A and B).
and cells transfected with ActRII and ALK4 (290 pM; Fig. 5B), whereas the ability of mutant M91E to displace 125I-activin-A from 293T cells transfected with ActRII was severely compromised. These results, coupled with the functional studies, indicate that substantial changes, such as the introduction of charged residues, in the finger region of activin-A disrupt binding to both ActRII and ALK4. The K102E mutant that had previously been shown to have disrupted binding to ActRII was included as a control in these experiments (Fig. 3B) (24).

**Biological Activity of the M108A Mutant**—The mutant that appeared to have the most favorable properties based on the initial functional screen and the competition binding assay was the M108A analog. This was due to its low activity (~8% of wild-type at a 2 nM dose) in the functional assay and its high affinity for ActRII in the competition binding assay (180 pM). To further characterize the behavior of the M108A mutant, we performed luciferase assays in 293T cells comparing the dose response relationships of wild-type activin with that of the M108A variant. At concentrations below 25 nM the M108A variant exhibited clearly reduced activity compared with wild-type activin (Fig. 4). However, the activity of the M108A mutant increased significantly when assayed at concentrations approaching 100 nM (Fig. 4). This suggested that M108A may act as a full, albeit very weak, activin agonist. Interestingly, the differences observed in the potencies of wild-type activin and the M108A mutant (over 3 orders of magnitude, Fig. 4) were not reflected in their comparable affinities for ActRII (Fig. 3B). To help explain this discrepancy we examined the contribution of ALK4 to activin affinity. In non-transfected 293T cells (Fig 5A), or cells transfected with both ActRII and ALK4 (Fig. 5B), the apparent affinity of activin was 60–80 pM, or 2-fold higher than the affinity observed in cells transfected with ActRII alone (150 pM). In contrast, the affinity of the M108A mutant for both non-transfected 293T cells (370 pM; Fig. 5A) and cells transfected with ActRII and ALK4 (290 pM; Fig. 5B), was 2–3-fold lower than the affinity observed in cells transfected with ActRII alone (150 pM; Fig. 3B). These results demonstrate for the first time that ALK4 does contribute to the affinity of activin for its receptor complex and suggest that the ability of the M108A mutant to associate with ALK4 is compromised.

**Covalent Cross-linking of Mutant M108A to ActRII and ALK4**—Based on the competitive binding and functional assays, we predicted that the M108A mutant would have a substantially reduced ability to bind ALK4 making it a dominant negative form of activin. To test this directly, our aim was to express and purify microgram quantities of M108A for iodination and covalent cross-linking studies. To express large quantities of M108A and other activin-A analogs, PEI was used to transfect 293T cells in 15-cm plates and conditioned media was harvested 72 h post-transfection. This method proved to be highly adaptable to the activin system with 1–4 μg of the M108A mutant produced per 15-cm plate.

To confirm that mutant M108A exhibited compromised ALK4 binding, we transfected cells with ActRII and ALK4, and performed affinity labeling experiments. Fig. 6A shows that when 293T cells were transfected with ActRII (Fig. 6A, lane 1) and then subjected to labeling and cross-linking with 125I-activin-A followed by immunoprecipitation with an antibody directed against ActRII, an activin-ActRII cross-linked complex of ~80 kDa was evident consistent with previous cross-linking results (25). The appearance of two activin-ActRII bands is routinely observed and is likely the result of differential glycosylation of ActRII (20). Co-transfection of ActRII with ALK4 resulted in cross-linking of 125I-activin-A to both receptor types as indicated by the appearance of the activin-ALK4 cross-linked complex at ~70 kDa. The ALK4 band was observed following immunoprecipitation with an antibody against either ActRII (Fig. 6A, lane 2) or ALK4 (Fig. 6A, lane 3).

Fig. 6B shows that when 293T cells were transfected with ActRII (Fig. 6B, lane 1) and then subjected to labeling and cross-linking with 125I-M108A followed by immunoprecipitation with an antibody directed against ActRII, an activin
(M108A)-ActRII cross-linked complex of ~80 kDa was evident similar to that seen with wild-type labeled activin-A. However, co-transfection of ActRII with ALK4 did not result in cross-linking of 125I-M108A to the type I receptor as the ALK4 band was not visible following immunoprecipitation with antibodies directed against ActRII-myc or ALK4 (Fig. 6B, lanes 2 and 3) as had been seen with wild-type 125I-activin-A. These results are consistent with the inability or greatly reduced ability of the M108A mutant to bind ALK4.

**Mutant M108A Antagonizes Activin and Myostatin Signaling**—The effect of the M108A mutant on activin-A induction of luciferase activity in 293T cells was measured. Fig. 7A shows that cells transfected with A3-luciferase and FAST2 and then treated with 25 nM M108A showed a relatively small (~11-fold) induction of luciferase activity that was substantially less than that seen in response to 100 pM activin-A (~48-fold). The addition of increasing amounts of M108A in the presence of a constant dose of wild-type activin-A (100 pM) caused a significant decrease in the activin-A induced luciferase response (from ~48- to ~23-fold). We also assessed the effect of M108A on the ability of myostatin (GDF8) to induce a luciferase response in 293T cells (Fig. 7B). These cells are somewhat less sensitive to myostatin than to activin (data not shown), however, treatment with 500 pM myostatin induced a 58-fold increase in luciferase activity. The addition of increasing doses of M108A in the presence of a constant dose of myostatin (500 pM) caused a significant reduction in the myostatin-induced luciferase response (from ~58 to ~26-fold). As with activin, the inhibitory effects are somewhat larger than they appear due to the small intrinsic signaling activity of the M108A mutant. These data indicate that the M108A mutant can block activin and myostatin signaling via type II activin receptors in 293T.
cells and suggests that this activin-A analog may be generally capable of antagonizing ligands that signal via type II activin receptors.

TGF-β does not signal via ActRII/IIB but rather utilizes its own type II receptor (TβRII). Therefore, to examine whether M108A specifically antagonizes activin type II receptors, we determined the effect of M108A on TGF-β induction of luciferase activity in 293T cells. As is the case with activin, transfection of FAST2 into 293T cells generates a system that is very sensitive to TGF-β stimulation. Fig. 7C shows that, in contrast to its effects on activin-A and myostatin signaling, addition of increasing doses of M108A to a constant amount of TGF-β (50 ng/ml) led to an apparent increase in TGF-β-induced activity (from 32–46-fold). This increase most likely reflects the small amount of residual activin activity observed for M108A (−11-fold).

Regardless, this result demonstrates that M108A does not antagonize TGF-β signaling in 293T cells and is consistent with its proposed action as a selective antagonist of type II activin receptors.

Antagonism of Activin-induced FSH Release from LβT2 Cells by Mutant M108A—In order to analyze the antagonist activity of the M108A mutant in more detail we determined its ability to block the activin-induced release of FSH from LβT2 cells (Fig. 8A). Increasing doses of M108A decreased basal FSH secretion from 17 to 6 ng over 72 h, presumably by affecting the action of locally secreted activin (26). In addition, the M108A mutant attenuated the stimulation of FSH secretion induced by increasing doses of exogenous activin-A (Fig. 8B).

**DISCUSSION**

The characterization of the activin-ALK4 binding interface could provide new insights into the primary steps and mechanism of activin receptor activation. Recently, we showed that there is only a partial overlap of the binding sites on ALK4 and ALK3 for activin-A and BMP2, respectively (20). We were interested to determine whether the type I receptor binding epitopes on the ligands were also distinct. In the structure of BMP2 in complex with ALK3, the two receptor molecules each bind to one of the two “wrist” epitopes of the BMP2 dimer in such a way that each ALK3-ECD makes contacts with both BMP2 monomers (18). The wrist epitope of BMP2 is formed by residues of the pre-helix loop and α-helix of one monomer and contacts the inner concave surface of the fingers of the other monomer. Important residues in the wrist epitope of BMP2 for ALK3 binding include; Phe49, Pro50, Leu51, Ala52, Asp53, His54, Asn55, Ile62, Val63, and Leu66 from one monomer; and Trp28, Trp31, Met89, Tyr103, and Met106 from the second monomer (18).

Based initially on the crystal structure of the BMP2-ALK3-ECD complex, and subsequently on information obtained from the structure of activin-A bound to ActRIIB, we subjected activin-A to alanine and homology scanning mutagenesis in an effort to identify the amino acids required for ALK4 binding and function. In order to screen the resulting 38 mutants, we utilized a rapid luciferase-based assay in HEK293T cells (22). Our results indicate that there are some significant differences in the binding sites on activin-A and BMP2 for ALK4 and ALK3, respectively. Mutation to alanine of residues in the pre-helix loop and α-helix of activin-A had very little, if any, effect on biological activity. Even combined amino acid substitutions, such as replacing residues Pro45, Ser46, His47, and Ile48 with alanines, did not decrease the activin-induced luciferase response. In contrast, a similar combinatorial mutation in the pre-helix region of BMP2 (F49A/P50A) decreased biological activity to less than 0.5% of the wild-type protein (27).

Only substantial mutations, such as exchanging the entire α-helix of activin-A for that of the activin-C analog (A/C-2 mutant), had significant effects on activin-A activity. Activin-C was chosen to generate a chimera with activin-A based on its reported lack of biological activity (28) and the fact that the two proteins share only 2 of 13 residues through the α-helix. Subsequent studies have indicated that activin-C may be biologically active (29), however, in the context of the activin-A molecule the activin-C α-helix was unable to confer activity. Similarly, the insertion of rigid proline residues at specific points in the α-helix (S60P, I63P) was sufficient to significantly decrease activin-A activity. Proline residues disrupt α-helices and the low activity (~20% of wild-type activin-A) of mutants S60P and I63P suggested that the α-helix was indeed important for activin-A function. However, mutation to proline of adjacent residues (Ser57, His59, Thr61, Val62) had limited effects on activin-A activity indicating that, although some residues in the α-helix are involved in receptor binding, this binding is not dependent upon the integrity of the α-helix.

Of greater importance for the biological activity of activin-A were residues on the concave (Met91, Ile105, Met108) and convex (Ser90, Leu92, Lys102) faces of finger 2. Residues Ser90, Leu92, and Lys102 form part of the type II receptor binding epitope on activin-A and were included in this study as controls (16, 17). Residues Met91, Ile105, and Met108 have not previously been implicated in binding to activin receptors, however, the corresponding residues on BMP2 (Met89, Tyr103, Met106), in combi-
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withation with several α-helical residues, form a hydrophobic pocket that accommodates a phenylalanine residue on ALK3 (18). Mutation of Met\(^{156}\) to alanine or glutamic acid significantly disrupted activin-A signaling activity (8 and 3% of wild-type activity, respectively, at a 2 nM dose). In contrast, alanine substitutions at residues Met\(^{91}\) and Ile\(^{105}\) had little effect on activin-A activity, and it was only with the introduction of charged residues at these sites that significant disruption was achieved.

In the present study we have shown that residues from both these regions are important for activin-A binding and activity. The identification of activin mutants with disrupted ALK4 binding allowed us to differentiate between activin binding to ActRII alone or to ActRII in the presence of ALK4. Previous studies had suggested that ALK4 does not contribute to activin affinity (30, 31), however, we found that the affinity of activin-A increased 2-fold in cells co-transfected with ActRII and ALK4 compared with cells transfected with ActRII alone. In contrast, the affinity of the M108A mutant actually decreased 2–3-fold in cells co-transfected with ActRII and ALK4 compared with cells transfected with ActRII alone. Interestingly, it was recently reported that Thickveins, the Drosophila type I BMP receptor, induced a 2-fold increase in the affinity of the ActRII-ECD for BMP2, and that the ActRII-ECD caused a 5-fold increase in the affinity of the ALK2-ECD for BMP7 supporting a role for cooperativity in receptor assembly (17). The affinities obtained for activin-A and M108A in the transfected system corresponded with those obtained in non-transfected cells and suggested that, in the presence of ALK4, the M108A mutant has a 5-fold lower affinity than wild-type activin-A.

Interestingly, this 5-fold shift in the ability of the M108A mutant to bind cells expressing both ActRII and ALK4 corresponds to a dramatic loss in the potency of this activin variant. A similar finding has been described for mutations in the knuckle epitope of BMP2 (27). In this study, the binding of BMP2 mutants A34D and H39D/S88A to the BMPRII-ECD was reduced only 10\(^{-5}\) fold in the affinity of the ALK2-ECD for BMP7 supporting a role for cooperativity in receptor assembly (27). The authors speculated that the simultaneous binding of two type II receptor chains is necessary for efficient receptor activation (32) and that a decrease in binding affinity becomes amplified when assessing function (27). A similar explanation could explain the very low potency of the M108A mutant. In this case, the simultaneous binding of two ALK4 molecules may be required for effective receptor activation and the decrease in M108A affinity for ALK4 would lead to an exaggerated decrease in activity.

Based on its low functional activity and its high affinity for ActRII (~180 pM) the M108A mutant was pursued as a candidate activin type II receptor antagonist. Signaling in 293T cells in response to both activin and the related ligand myostatin was reduced in a dose dependent manner by the M108A variant. At the same time, M108A was unable to antagonize TGF-β signaling consistent with its proposed action as a selective antagonist of type II activin receptors. In a more physiological assay, we tested the ability of M108A to block activin-induced FSH secretion from a gonadotrope cell line. Previous studies have shown that L6/T2 cells express the components of the activin system, and that addition of follistatin alone reduces FSH\(^{β}\) gene expression, suggesting that an endogenous activin autocrine loop regulates FSH in these cells (26). Our finding of a relatively high basal FSH tone in these cells that was significantly reduced in the presence of increasing doses of the M108A mutant supports these earlier studies. Moreover, the M108A mutant interfered with the ability of exogenous activin-A to stimulate FSH secretion.

One unexpected aspect of the antagonism experiments was that the M108A mutant could not completely attenuate the luciferase response induced by activin or myostatin. This can be explained, in part, by the fact that the M108A mutant is not a full antagonist but rather has agonist activity at high doses. Residual signaling activity of the mutant apparently prevented a complete block of the activin response. The dose of the M108A mutant required to block activin and myostatin in the luciferase assay was also higher than predicted based on its ability to bind ActRII with high affinity. This may reflect the fact that activin utilizes a multicomponent receptor system to signal. In this case, the ability of the M108A mutant to antagonize activin signaling is dependent upon its ability to block activin access to its four-receptor complex, not just to ActRII.

The generation of activin-A antagonists in this study was most likely a consequence of the ordered sequential binding mechanism operating during receptor activation. The M108A mutant has an intact knuckle epitope and retains the ability to bind ActRII with high affinity. However, the disruption of the wrist epitope limits subsequent interaction of M108A with the low affinity type I receptor ALK4. The promiscuity of the activin type II receptors suggests that antagonists based on activin mutants may be generally capable of antagonizing ligands that signal via ActRII/IB. Indeed, the antagonism of myostatin signaling by the M108A mutant supports this proposal. Because the multiple ligands that signal via activin type II receptors control the physiologic behavior of virtually all organ systems, targeting these receptors with selective modulators or antagonists provides the opportunity for therapeutic intervention in many human diseases including muscular dystrophy (33), cachexia (34, 35), wound healing (36, 37), liver regeneration (38), and cancer (8).

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