Activin A/Bone Morphogenetic Protein (BMP) Chimeras Exhibit BMP-like Activity and Antagonize Activin and Myostatin

Radhika V. Korupolu, Uwe Muenster, Jessica D. Read, Wylie Vale, and Wolfgang H. Fischer
From the Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla California 92037

Activins and bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β family of growth and differentiation factors that induce signaling in target cells by assembling type II and type I receptors at the cell surface. Ligand residues involved in type II binding are located predominantly in the C-terminal region that forms an extended β-sheet, whereas residues involved in type I binding are located in the α-helical and preceding loop central portion of the molecule. To test whether the central residues are sufficient to determine specificity toward type I receptors, activin A/BMP chimeras were constructed in which the central residues (45–79) of activin A were replaced with corresponding residues of BMP2 and BMP7. The chimeras were assessed for activin type II receptor (Act RII) binding, activin-like bioactivity, and BMP-like activity as well as antagonistic properties toward activin A and myostatin. Acta/BMP7 chimera retained Act RII binding affinity comparable with wild type activin A, whereas Acta/BMP2 chimera showed a slightly reduced affinity toward Act RII. Both the chimeras were devoid of significant activin bioactivity in 293T cells in the A3 Lux reporter assay up to concentrations 10-fold higher than the minimal effective activin A concentration (~4 nM). In contrast, these chimeras showed BMP-like activity in a BRE-Luc assay in HepG2 cells as well as induced osteoblast-like phenotype in C2C12 cells expressing alkaline phosphatase. Furthermore, both the chimeras activated Smad1 but not Smad2 in C2C12 cells. Also, both the chimeras antagonized ligands that signal via activin type II receptor, such as activin A and myostatin. These data indicate that activin residues in the central region determine its specificity toward type I receptors. Acta/BMP chimeras can be useful in the study of receptor specificities and modulation of transforming growth factor-β members, activins, and BMPs.

Activins and BMPs are members of the TGF-β2 superfamily that comprises >40 encoded members in the human. At least five binding receptors (RII) and seven signaling receptors (RI) that bind the ligands of the family have been identified (1–3). The members of this superfamily are involved in a multitude of biological functions including development, differentiation, apoptosis, reproduction, tissue regeneration, immune responses, and bone growth (4–7).

The ligands of TGF-β family share a distinct structural signature known as the “cysteine knot scaffold” (8). Activins adopt this prototypical disulfide-linked dimeric structure and contain two subunits termed β-chains. Genes encoding four different β subunits, βA, βB, βC, and βE, have been identified in the human, theoretically offering a possibility of an array of activin dimers, although only activin A (βA-βA), activin-B (βB-βB), and activin AB (βA-βB) have been documented to be biologically active (9). In each monomer two pairs of antiparallel β-strands stretch out from the cysteine core of the dimer to form short and long fingers. The characteristic curvature of these fingers creates concave and convex surfaces on the ligand. At the base of the fingers, each monomer has an α-helix, which together with the pre-helix loop and the inner concave surface of the fingers of the other monomer form the “wrist” region (10, 11).

Bone morphogenetic proteins (BMPs) were first identified as molecules that induce bone and cartilage formation in rodents (12). BMPs are a large family (more than 20 members) with extremely complex and diverse roles both in development and adult life (12–14). For example, BMP7 has a role in kidney morphogenesis and bone formation during development and is involved in regulating gonadal function in the adult (15–18). BMPs, like activins, signal via type II and type I receptors (19) and subsequently activate Smad proteins 1, 5, and 8, which in turn transmit signals into the cell nucleus (6, 20). Although BMP type I receptors (ALK3, ALK6, and ALK2) are largely specific for BMP family, it is not true for type II receptors. BMP RII binds only BMPs (21–23), but activin type II receptors can bind both BMPs and activins (24).

The receptor activation mechanism for activin involves initial binding to its type II receptor (Act RII or IIB), which leads to the recruitment, phosphorylation, and activation of its type I receptor (ALK4) followed by activation of intracellular signaling molecules, Smad2 and -3 (25–27). The specific amino acid residues of activin A involved in the underlying intermolecular interactions within the activin/Act RII-ECM/ALK4-ECM complex can be deduced now based on relevant structural and functional data. The activin A/Act RIIb interface involves hydrophobic (Ile-30, Ala-31, Pro-32, Pro-88, Leu-92, Tyr-94, and

This is an Open Access article under the CC BY license.

JOURNAL OF BIOLOGICAL CHEMISTRY 3782 VOLUME 283 • NUMBER 7 • FEBRUARY 15, 2008
**MATERIALS AND METHODS**

SDS-PAGE gels (4–12%) used were from Bio-Rad, 125I-labeled activin A was prepared using chloramine T method as described previously (33) by Ezra Wiater, Peptide Biology Laboratories, Salk Institute, La Jolla, CA. The ActA/BMP constructs used in this study were in the pCDNA3 expression vector (Invitrogen). Recombinant BMP2, BMP7, and myostatin were purchased from R & D Systems (Minneapolis, MN). Phospho-Smad1 and Phospho-Smad2 antibodies were purchased from Cellular Signaling Technologies (Beverly, MA).

**Construction of Chimeras**—Chimeras were generated by two-step introduction of BMP base pairs into the activin A sequence. First, plasmids containing sequences for ActA/BMP2 45–52, ActA/BMP7 73–79, ActA/BMP2 45–52, and ActA/BMP7 73–79 were generated by “long PCR” (initial denaturation, 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; final extension, 10 min at 72 °C) using pCDNA containing the sequence for each ActA/BMP2 45–52, ActA/BMP7 73–79, ActA/BMP2 45–52, and ActA/BMP7 73–79 as template together with NheI pr or XhoI pr, respectively, as well as primers introducing base pairs encoding for BMP2 or BMP7 residues 53–72, two pieces of overlapping DNA were generated for each chimera. In a second PCR (initial denaturation, 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; and final extension, 10 min at 72 °C), the two overlapping DNAs of BMP2 (to generate ActA/BMP2 45–79) as well as the two overlapping DNAs of BMP7 (to generate ActA/BMP7 45–79) were combined, and NheI pr or XhoI pr, respectively, as well as primers introducing base pairs encoding for BMP2 or BMP7 residues 53–72, two pieces of overlapping DNA were generated for each chimera. In a second PCR (initial denaturation, 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; and final extension, 10 min at 72 °C), the two overlapping DNAs of BMP2 (to generate ActA/BMP2 45–79) as well as the two overlapping DNAs of BMP7 (to generate ActA/BMP7 45–79) were combined, and NheI pr or XhoI pr were used as primers. The resulting products were cut with NheI and XhoI and ligated into the pCDNA cassette described above. All of the PCRs were performed using 2.5 units of Takara DNA polymerase (Takara, Madison, WI) along with 0.2 unit of Pfu polymerase (Stratagene, La Jolla, CA). PCR products were separated on 1% agarose gels (Bio-Rad). To amplify the constructs, the plasmids were transformed into Top 10 competent bacteria by chemical transformation. Mini- and maxipreps as well as gel purifications were carried out using Qiagen (Valencia, CA) kits.

**Expression and Purification of Chimeric Proteins**—For protein expression, ActA/BMP2 and ActA/BMP7 chimeric plasmids were transfected into 293T cells using polyethyleneimine as described (34). In brief, 293T cells were grown to 70–80% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and l-glutamine in polylysine-coated 15-cm cell culture plates. After removal of the media, cells were washed with serum-free media, and 11 ml of serum-free medium was added to the cells. A solution of 36 μg of polyethyleneimine and 24 μg of plasmid DNA in 1.2 ml of serum-free medium was prepared, incubated for 10 min at room temperature, and then added to each cell culture dish. Cells were grown at 37 °C, 5% CO2. After 3 h, fetal calf serum was added to the cells to a final concentration of 10%. After 72 h, crude media containing chimeric proteins were harvested and filtered through a 5-μm nylon filter to separate cell debris. Then 1 mM MES buffer, pH 6.2, was added to the filtrate to a final concentration of 50 mM along with 0.5 ml of M2 anti-FLAG-agarose bead suspension (Sigma). The media were left overnight at 4 °C under mild shaking conditions to allow protein binding to the beads. Then the suspension was poured into columns (10 cm × 1 cm; Bio-Rad) equipped with a one-way stop cock, the flow-through and 15 ml of additionally added 50 mM MES washing buffer were discarded, and ActA/BMP chimeras bound to the M2 anti-FLAG-agarose beads were eluted upsteam of the N-terminal FLAG sequence (Nhel pr) together with a reverse primer containing an Xhol site annealing to the C terminus of the chimeras (Xhol pr). The resulting products were cut with Nhel and Xhol and then ligated overnight at 16 °C into an Nhel-Xhol cut pCDNA cassette containing the remaining wt activin A sequence. To obtain the cassette an Nhel site was introduced by silent mutation 169 bp upstream of the mature activin A in pCDNA. The Act/A/BMP2 45–79 and ActA/BMP7 45–79 chimeras were constructed by “overlapping PCR” using the above produced plasmids as templates. In four separate PCRs (initial denaturation, 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; final extension, 10 min at 72 °C) using pCDNA containing the sequence for either ActA/BMP2 45–52, ActA/BMP7 73–79, ActA/BMP2 45–52, and ActA/BMP7 73–79 as template together with Nhel pr or XhoI pr, respectively, as well as primers introducing base pairs encoding for BMP2 or BMP7 residues 53–72, two pieces of overlapping DNA were generated for each chimera. In a second PCR (initial denaturation, 5 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; and final extension, 10 min at 72 °C), the two overlapping DNAs of BMP2 (to generate ActA/BMP2 45–79) as well as the two overlapping DNAs of BMP7 (to generate ActA/BMP7 45–79) were combined, and Nhel pr as well as XhoI pr were used as primers. The resulting products were cut with Nhel and XhoI and ligated into the pCDNA cassette described above. All of the PCRs were performed using 2.5 units of Takara DNA polymerase (Takara, Madison, WI) along with 0.2 unit of Pfu polymerase (Stratagene, La Jolla, CA). PCR products were separated on 1% agarose gels (Bio-Rad). To amplify the constructs, the plasmids were transformed into Top 10 competent bacteria by chemical transformation. Mini- and maxipreps as well as gel purifications were carried out using Qiagen (Valencia, CA) kits.
Activin/BMP Chimeras Exhibit BMP-like Activity

Transfection and Luciferase Assays in HepG2 Cells—HepG2 cells were grown at 37 °C in a 5% CO₂ humidified incubator in α-modification of Eagle’s medium (Fisher Mediatech, Pittsburgh, PA) supplemented with 10% fetal bovine serum and L-glutamine. Cells grown in 24-well plates (surface area ~1.75 cm²) were transfected with the bone morphogenetic protein-responsive BRE-Luc reporter plasmid (35), Rous sarcoma virus β-galactosidase, and pCDNA3 empty vector in a ratio of 0.9 μg/0.1 μg/0.05 μg using SuperFect transfection reagent (Qiagen) according to the manufacturer’s recommendations. After 24 h, fresh medium was added, and cells were treated with increasing doses of activin A, BMP-2, BMP-7, ActA/BMP2 chimera, or ActA/BMP7 chimera as indicated for 16–24 h. Then cells were harvested in solubilization buffer (1% Triton X-100, 25 mM HEPES, pH 7.8, 15 mM MgSO₄, 5 mM EGTA), and luciferase reporter activity was measured and normalized to β-galactosidase activities using standard methods.

Transfection and Luciferase Assays in HEK 293T Cells—293T cells were seeded into 24-well plates coated with polysyline at a density of 150,000 cells/well. After 24 h cells were transfected overnight with a mixture of A3 Lux (25 ng) and β-galactosidase (25 ng) reporter plasmids, the transcription factor FAST2 (50 ng), and empty pCDNA3 vector (400 ng) using Perfectin® transfection reagent (GenLantis) according to the manufacturer’s recommendations. Then the cells were treated with increasing doses of wt activin A or ActA/BMP chimeras for 16–24 h. The cells were harvested in ice-cold lysis buffer (1% Triton X-100 in 25 mM glycyglycine, 4 mM EGTA, 15 mM MgSO₄ containing 1 mM dithiothreitol) and assayed for luciferase and β-galactosidase activities using standard methods. To assess antagonistic properties of the ActA/BMP chimeras, the cells were treated alternatively either with 100 pM wt activin A or 500 pM myostatin in the presence of increasing doses of the chimeras for 16–24 h.

Inhibition of Follicle Stimulating Hormone (FSH) Release from Rat Interior Pituitary Cells—The assay was performed as previously described (32). Briefly, freshly isolated cells from male Sprague-Dawley rat interior pituitaries from several animals were combined and seeded into 96-well plates at a density of 50,000 cells/well in BMP medium (as detailed in Ref. 36) supplemented with 2% fetal bovine serum and appropriate growth factors (36). After 24 h cells were treated with increasing doses of wt activin A or ActA/BMP chimera (0–40 nM) in the presence or absence of 100 pM wt activin A. 72 h later media were harvested, and the concentration of the secreted FSH was determined by radioimmunoassay.

Differentiation and Alkaline Phosphatase Assays in C2C12 Cells—C2C12 cells were grown in 24-well plates coated with polysyline and seeded at a density of 150,000 cells/well were transfected with mouse Act RII using Perfectin® (GenLantis, San Diego, CA). After 48 h the cells were incubated with increasing doses of activin A or chimeras in the presence of a constant amount of iodinated wt activin A tracer (200,000 cpm/well) for 2 h in HDB (12.5 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl) containing 1.5 mM CaCl₂, 0.1% bovine serum albumin, and 5 mM MgSO₄. After incubation, binding buffer was removed, and the cells were washed 3 times with HEPES dissociation buffer and then lysed with 1% SDS solution for 20 min. Radioactivity in the cell lysates was measured using a γ counter (APEX Micromedic Systems, Horsham, PA).
**RESULTS**

Selection of the Region for Chimera Construction—Based on available structural information, residues involved in ALK3 binding of BMP2 are located in both wrist and finger regions of BMP2 (29). Recently two activin A mutants containing a point mutation in finger 2 (32) as well as a chimera, in which the entire wrist of activin A was changed to corresponding residues of activin C (A/C46–78) (31) were shown to retain their affinity for the type II receptor, Act RII. Concurrently, these mutants were devoid of activin-like activity and consequently possessed activin and myostatin antagonistic properties. Following our hypothesis that TGF-β family protein wrist residues determine type I receptor specificity, we constructed chimeras by exchanging the wrist region 45–79 of activin A to correspond to BMP2 and ActA/BMP7 chimeras. BMP2 residues implicated in ALK3 binding are shaded gray.

Expression and Purification of Activin A/BMP Chimeras—The anti-FLAG column purified ActA/BMP2 and ActA/BMP7 chimeras upon reverse phase HPLC on C4 column eluted off as single peaks at 46.3 and 45.1% acetonitrile, respectively. Western blots of respective peak fractions run under reducing conditions showed a single band at 19 kDa, whereas wt activin A standard appeared at 13 kDa. The difference in the size between chimeras and wt activin A is due to the FLAG tag attached on the N terminus of the chimeras and glycosylation. Silver staining of these fractions on a SDS gel proved to be near homogeneous (Fig. 2). Expression levels of the chimeric proteins ranged between 0.4 and 1 μg per 15-cm cell culture dish for both ActA/BMP2 and ActA/BMP7 chimeras.

Act RII Binding—Based on the previous available data through mutagenesis studies and crystal structures, ActA/BMP chimeras are predicted to retain Act RII receptor binding affinity (11, 28–31). Therefore, we started with assessing their ability to displace 125I-labeled activin A from 293T cells transfected with mouse Act RII cDNA. Displacement curves revealed IC50 values of 90–130, 270, and 120 pM for wt activin A, ActA/BMP2, and ActA/BMP7 chimeras, respectively, indicating that the chimeras retained their affinity for Act RII (Fig. 3).

---

**FIGURE 1.** Sequence alignment of activin A, BMP2, and BMP7. The mature, human activin A, BMP2, and BMP7 protein sequences were aligned using the Megalign program. Activin A residues exchanged with BMP2/BMP7 residues are boxed. Activin A residues involved in Act RII binding are shown in white letters on black. BMP2 residues implicated in ALK3 binding are shaded gray.

**FIGURE 2.** Protein purification. HPLC fraction collected at 35.1 min for ActA/BMP2 and 33.4 min for ActA/BMP7 purification were subjected to 4–12% gradient SDS-PAGE under reducing and non-reducing conditions, blotted onto polyvinylidene difluoride membrane, and treated with primary antibodies raised against activin A residues 81–113 followed by treatment with appropriate alkaline phosphatase-conjugated secondary antibodies. The blot was then visualized using BCIP/NBT. Panel A, Western blot. Lane 1, ActA/BMP2 (reduced); lane 2, ActA/BMP7 (reduced); lane 3, wt ActA (reduced); lane 4, ActA/BMP7 (nonreduced); lane 5, ActA/BMP2 (nonreduced); lane 6, wt ActA (nonreduced). Panel B, silver-stained gel. Lane 1, wt ActA (reduced); lane 2, ActA/BMP2 (reduced); lane 3, ActA/BMP7 (reduced); lane 4, wt ActA (nonreduced); lane 5, ActA/BMP7 (nonreduced); lane 6, ActA/BMP2 (nonreduced).
Activin/BMP Chimeras Exhibit BMP-like Activity

Activin-like Bioactivity of Chimeras—Because the chimeras retained their type II binding as predicted, we continued to examine the activin-like bioactivity of the ActA/BMP chimeras. The activin-like activity was characterized by their ability to activate an A3 Lux reporter gene in HEK 293T cells. Wt activin A at a concentration of 20 nM led to a 32-fold induction of luciferase activity (EC50/200 pM). However, ActA/BMP chimeras did not stimulate the A3 Lux reporter in concentrations up to 40 nM, suggesting that the chimeras lost their ability to signal in an activin-like manner (Fig. 4).

BMP-like Activity of Chimeras—The residues in the wrist region of BMP2 are believed to be involved in its type I receptor binding (29); we thus predicted the chimeras to signal via BMP type I receptors acquiring BMP-like activity. Therefore, the BMP-like activity of the chimeras was assessed by characterizing their ability to activate BRE-Luc in HepG2 cells. The hepatocyte cell line HepG2 is a relevant model system because BMPs are expressed in liver, are proposed to maintain the local liver homeostasis (38), and are involved in liver development (39). Additionally, HepG2 cells express both Act RII and BMP type I receptors and, thus, are BMP-responsive (39). In contrast to wt activin A, wt BMP2 and wt BMP7 stimulated BRE-Luc in HepG2, indicating that BRE-Luc activation is BMP-specific (Fig. 5, A and B). wt BMP7 at a concentration of 40 nM led to a 23-fold BRE-Luc induction (EC50, 5 nM), whereas wt BMP2 at a concentration of 40 nM induced BRE-Luc 12-fold (EC50, 4 nM). Also, the ActA/BMP chimeras stimulated the BRE-Luc in HepG2 cells with EC50 values of 10 nM for ActA/BMP2 chimera and 1 nM for ActA/BMP7 chimera (Fig. 5, A and B). These data indicated that the chimeras signal in BMP-like manner.

To further confirm that ActA/BMP chimeras have BMP-like activity in an appropriate cellular context, we tested for BMP-like responses in the mouse myoblastic cell line, C2C12. Under low serum conditions, C2C12 cells differentiate into a myotube-like phenotype. BMPs shift differentiation to osteoblast-like cell expressing alkaline phosphatase (37). As shown in Fig. 5C, 10 nM activin A could not induce significant expression of alkaline phosphatase, whereas 10 nM wt BMP2 and 10 nM wt BMP7 expressed significant amount of alkaline phosphatase. The ActA/BMP2 chimera (10 nM) also expressed significant amounts of alkaline phosphatase, whereas 10 nM ActA/BMP7 had a weak response corroborating the results obtained with the BRE-Luc assay in HepG2 cells. Indeed, under differentiating conditions activin A had no significant effect on myotube formation, whereas wt BMPs and ActA/BMP chimeras shifted the...
differentiation into a monolayer of osteoblast-like cells (Fig. 5). These data clearly established that the chimeras have switched toward BMP type I receptor from activin type I receptor.

Smad Activation by Chimeras—The Smads are the established intracellular effectors of TGF-β family signaling. Upon ligand binding, the activin type I receptors recruit and phosphorylate Smad2 and 3 proteins, whereas the BMPs phosphorylate Smad1, -5, and -8. Because the ActA/BMP chimeras are presumed to bind BMP type I receptor instead of activin type I receptor, they should phosphorylate Smad1, -5, and -8 but not Smad2 and -3. To confirm this, we checked for the phospho-Smad2 and phospho-Smad1 levels in C2C12 cells after treatment with wt activin A or wt BMP2/7 or ActA/BMP chimeras. As predicted, on treatment with 5 nM activin A, the C2C12 cells showed a substantial increase in phospho-Smad2 at 30 min but not phospho-Smad1, whereas the wt BMP2/7 showed an increased phospho-Smad1 but not phospho-Smad2 (Fig. 6). ActA/BMP2 chimer showed a significant increase in phospho-Smad1 levels even at concentrations as low as 1 nM, whereas the ActA/BMP7 showed a slight increase in phospho-Smad1 at 5 nM concentration corroborating the results showing lower BMP-like activity in HepG2 cells and C2C12 cells for ActA/BMP7 chimera (Fig. 6). No changes in levels of total Smad2 and Smad1 were noted over the 60-min treatment period (data not presented). These data clearly indicated that the ActA/BMP chimeras activated the Smad proteins specific for BMPs.

Activin A/BMP Chimeras Antagonize Activin A and Myostatin—Because the ActA/BMP chimeras bind to Act RII and do not signal in an activin-like manner, they represent potential antagonists of the proteins that signal via Act RII/ALK4 pathway like the activin and myostatin. Therefore, we determined the ability of the chimeras to block activin A and myostatin-induced A3 Lux reporter in HEK 293T cells. Both chimeras indeed inhibited A3 Lux reporter induction in a concentration-dependent manner (Fig. 7). IC_{50} ranges for the inhibition of luciferase activity induced by 100 pM activin A were 1–8 nM (Fig. 7A) and 1–10 nM (Fig. 7B) for ActA/BMP7 and ActA/BMP2, respectively. Also, luciferase activity induced by 500 pM myostatin was reduced with a IC_{50} range of 1–5 nM (Fig. 7A) and 1–5 nM (Fig. 7B) for ActA/BMP7 and ActA/BMP2, respectively. We observed that the chimeras can block TGF-β1 also (data not shown), and this may be because of activation of Smads 1, 5, and 8 through BMP type I binding (BMP-like activity), which are known to have a negative effect on TGF-β bioactivity (40).

![Image](https://via.placeholder.com/150)

**FIGURE 5. BMP-like activity of chimeras.** Panels A and B, HepG2 cells transfected with BRE-Luc and β-galactosidase were treated with increasing doses of different proteins as shown, harvested, and assayed as described under “Materials and Methods.” Dose-response graphs for wt BMP7 (closed square), wt activin A (closed triangle), and ActA/BMP7 chimera (open square) (A) and wt BMP2 (closed circle), wt activin A (closed triangle), and ActA/BMP2 chimera (open circle) (B) were generated using Prism software. Curves represent the luciferase activity normalized to β-galactosidase activity. Each point on the graph is a mean ± S.D. of three measurements. The experiment was repeated at least three times. Panels C and D, C2C12 cells were treated with 10 nM wt activin A or wt BMP or ActA/BMP chimera as shown, grown in differentiation media for 5 days, and lysed. C, alkaline phosphatase activity was measured as described under “Materials and Methods.” **, p < 0.005; **, p < 0.001 (unpaired t test). D, cell morphology under low magnification (20×), bright field photography showed that cells have fused to myotubes (a and b) or are a monolayer of osteoblast-like cells (c, d, e, and f). The experiment was repeated at least three times. NT, No Treatment.
Activin/BMP Chimeras Exhibit BMP-like Activity

FIGURE 6. Smad activation by chimeras. C2C12 cells were treated with 1 or 5 nM wt activin A or wt BMP or ActA/BMP chimera for the indicated times. Phospho-Smad2 and phospho-Smad1 levels were determined using the appropriate antibodies by Western blotting. Bands were detected by horse-radish peroxidase chemiluminescence and exposure to BioMax Light Kodak films. The experiment was repeated at least three times.

To further substantiate the antagonistic properties of ActA/BMP chimeras, their inhibitory effect on basal and activin-induced FSH release from rat anterior pituitary cells in primary cell culture was tested. Rat anterior pituitary cells respond to activin by releasing FSH in vivo and are critical for the control of gonadal functions. Thus, this system is physiologically more relevant than the A3 Lux assay in 293T cells. Rat anterior pituitary cells were incubated in the presence or absence of 100 pM wt activin A with increasing doses of ActA/BMP2 or ActA/BMP7 chimera. After 72 h of incubation, the amount of FSH released was estimated by radioimmunoassay. Figs. 7C–E, show that both chimeras inhibit basal as well as activin-induced FSH secretion from rat anterior pituitary cells in a concentration-dependent manner. Basal FSH secreted into the rat anterior pituitary cell medium decreased from ~25 to 5 ng/ml and ~15 ng/ml when cells were incubated for 72 h with 40 nM Acta/BMP7 and Acta/BMP2 chimera, respectively (Fig. 7C). When cells were treated with 100 pM activin A in the presence of increasing doses of ActA/BMP chimera, FSH release induced by 100 pM activin A was brought back to basal level with 4 nM Acta/BMP7 chimera (Fig. 7D). To achieve the same effect, a 40 nM concentration Acta/BMP2 chimera was required (Fig. 7E). Interestingly, the Acta/BMP7 chimera inhibited FSH to a significantly lower level than basal value when concentrations of 13 nM and 40 nM (Fig. 7D) were added.

DISCUSSION

Recently, we showed that exchanging the entire wrist region of activin A with biologically inactive activin C blocks its type I receptor binding while retaining its Act RII binding (31). Concurrently, this activin A/C chimera acted as an antagonist to molecules that signal via the Act RII receptor. To answer the question of whether changing the entire wrist between the different members of TGF-β family allows construction of chimeras with desired type II and type I receptor specificities, we exchanged the entire wrist region of activin A with the corresponding BMP2 or BMP7 regions to examine if this would switch its signaling via activin type I to BMP type I receptors while retaining the affinity for Act RII. Indeed, both the ActA/BMP2 and ActA/BMP7 chimeras when characterized for Act RII binding retained their binding affinity comparable with that of wt activin A, in line with the predictions made based on crystal structures of activin A/Act RII (30) and Act RII/BMP7 as well as mutagenesis studies showing the outer surface, the “knuckle” of the finger region, to form the binding interface with the type II receptors (28, 30, 41). Based on the crystal structure of BMP2 bound to ALK3 (29), several residues in the BMP2 wrist (Phe-49, Pro-50, Leu-51, Ala-52, Asp-53, His-54, Ser-57, Ile-62, Val-63, and Leu-66 of the other monomer) are implicated in the binding interface. Several of these residues were confirmed to participate in ALK3 binding by mutagenesis studies (37, 42), showing especially the BMP2 mutants F49A, P50A, L51P, H54E, and double mutant F49A,P50A to possess a significantly decreased affinity for ALK3 as well as a reduced ability to induce alkaline phosphatase activity in C2C12 cells. Both Acta/BMP2 and Acta/BMP7 chimeras had significant BMP-like activity in a BRE-Luc assay as well as induced osteoblast-like phenotype and alkaline phosphatase in C2C12 cells showing that the wrist residues are important for BMP type I receptor binding interface. The fact that the BMP-like activity of these chimeras is reduced compared with their respective wt BMPs, even though they bind Act RII with affinity comparable with activin A indicates that there are some other weak interactions involving residues other than the wrist region in BMP2/7 interaction with its type I receptor. We attempted to quantify the affinity by competitive binding assay employing C2C12 cells transfected with BMP type I receptors, radiolabeled wt BMP, and increasing concentrations of the wt BMP or Acta/BMP chimera. The wt BMP tracer was not displaced significantly by either wt BMP or the Acta/BMP chimeras. A previous study by Koenig et al. (43) reported similar results and concluded that BMPs exhibit high nonspecific binding to whole cells and membrane preparations. Although the residues of the wrist region define specificity, residues outside this region are likely to strengthen the binding interactions. Along these lines, it was previously reported by Keller et al. (44) that BMP-2 (W28F) and BMP-2 (Y103A) mutants bind the BMP R-IA ECD with affinity lower by 3 and 8 times, respectively, than the wt BMP-2. These authors had to resort to surface plasmon resonance measurements with soluble BMP type I receptor ECDs to determine binding affinities. Replacement of the wrist region in activin A effectively reduced its signaling
through the activin type I receptor, indicating that the wrist loop is the most crucial region for its interaction with the type I receptor. Furthermore, ActA/BMP chimeras activated the BMP-specific Smads in contrast to activin A. Undoubtedly, introducing the wrist loop of BMP into activin A brings a switch in its type I receptor specificity and a subsequent change in specific Smad activation. This interesting observation can be useful in the study of the modulation of processes involving TGF-β family members.

Activin initially binds to its type II (Act RII/Act RIIB) receptor with high affinity and then to the type I receptor, ALK4 (26, 27). Mutants retaining wt-like Act RII/Act RIIB affinity but disrupted ALK4 binding should be antagonists to activin and any molecules that signal via activin type II and type I receptors. As predicted, ActA/BMP2 and Acta/BMP7 chimeras possessing affinity for Act RII comparable with wt activin A and at the same time devoid of significant wt activin-like bioactivity turned out to be antagonist for ligands signaling via Act RII and ALK4. These findings are in line with our previous reports on mutants of activin such as the M108A point mutant (32) and the activin A/C chimera (31). The antagonistic effect of Acta/BMP2 (IC50 1–8 nM) or Acta/BMP7 (IC50 1–10 nM) toward 100 pM activin A and 500 pM myostatin (IC50 for Acta/BMP2, 1–5 nM; IC50 for Acta/BMP7, 1–5 nM) appears similar to that reported for the activin A/C chimera (IC50, 1–10 nM) (31) and the activin (M108A) point mutant (32). The finding that the activin/BMP chimeras act as partial antagonists might be explained by overlapping intracellular signaling pathways (45). With ActA/BMP chimeras, the general principle to produce potential activin antagonists through blocking its type I receptor binding with intact type II receptor binding, as proposed by previous reports (31, 32), appears to be established. It needs further investigation to see if this general mechanism to produce antagonists can be extended to other members of the family, like TGF-β. Because many ligands that signal via activin receptors control the physiological 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, liver cirrhosis, wound healing, liver regeneration, and cancer.

**FIGURE 7. Antagonism of activin A and myostatin by chimeras.** Panels A and B, HEK293T cells were transfected with A3 Lux, β-galactosidase, FAST2, and treated with increasing doses of Acta/BMP7 or Acta/BMP2 chimera in the presence of 100 pM wt activin A or 500 pM myostatin for 16–24 h. Cells were lysed and assayed for luciferase and β-galactosidase activities as described under “Materials and Methods.” Dose-response curves for Acta/BMP2 + 100 pM activin A (closed triangles) and Acta/BMP7 + 500 pM myostatin (open circles) (A) and Acta/BMP2 + 100 pM activin A (closed triangles) and Acta/BMP2 + 500 pM myostatin (open circles) (B) were generated using Prism software. Curves represent the luciferase activity normalized to β-galactosidase activity. Each point on the graph is a mean ± S.D. of three measurements. The experiment was repeated at least three times. Panels C–E, rat anterior pituitary cells were treated with increasing doses of Acta/BMP7 or Acta/BMP2 chimera in the absence (C) or presence (D and E) of 100 pM activin A for 72 h. Media were harvested and assayed for released FSH by radioimmunoassay. Each point on the graph is a mean ± S.D. of three measurements. *, p < 0.05; **, p < 0.01 (one-way analysis of variance).
Activin/BMP Chimeras Exhibit BMP-like Activity

Acknowledgments—We thank Drs. E. Wiater, P. Gray, and K. Schmidt (The Salk Institute) for stimulating discussions. We thank Cindy Donaldson for isolation of rat anterior pituitary cells, FSH radioimmunoassay, and additional technical support. We thank Joan Vaughan for providing affinity purified primary antibodies against activin A residues 81–113.

REFERENCES