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Activins bind and signal *via* bone morphogenetic protein receptor type II (BMPR2) in immortalized gonadotrope-like cells

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ABSTRACT

TGFβ superfamily ligands greatly outnumber their receptors. Thus, receptors are shared between ligands and individual ligands can bind multiple receptors. Bone morphogenetic proteins (BMPs) bind and signal *via* both BMP type II (BMPR2) and activin type II (ACVR2) receptors. We hypothesized that, in addition to its canonical receptor ACVR2, activin A might similarly bind and signal *via* BMPR2. First, using surface plasmon resonance, we showed that activin A binds to the BMPR2 extracellular domain (ECD), though with lower affinity compared to the ACVR2-ECD. We confirmed these results in cells, where radiolabeled activin A bound to ACVR2 and BMPR2, but not to other type II receptors (AMHR2 or TGFBR2). Using homology modeling and site-directed mutagenesis, we identified key residues in BMPR2 that mediate its interaction with activin A. The soluble ECDs of ACVR2 or BMPR2 dose-dependently inhibited activin A-, but not TGFβ-induced signaling in cells, suggesting that activin binding to BMPR2 could have functional consequences. To address this idea, we altered BMPR2 expression levels in immortalized murine gonadotrope-like cells, LβT2, in which activins potently stimulate follicle-stimulating hormone β (*Fshb*) subunit transcription. BMPR2 expression potentiated activin A responses whereas depletion of endogenous BMPR2 with short interfering RNAs attenuated activin A-stimulated *Fshb* transcription. Additional data suggest, for the first time, that BMPR2 may form functional complexes with the canonical activin type I receptor, activin receptor-like kinase 4. Collectively, our data show that BMPR2, along with ACVR2, functions as a *bona fide* activin type II receptor in gonadotrope-like cells, thereby broadening our understanding of mechanisms of activin action.

1. Introduction

Transforming growth factor β (TGFβ) superfamily ligands regulate diverse biological processes, including cell proliferation and differentiation, apoptosis, organ development, wound healing, and reproduction [1–3]. In general, these ligands produce their effects by binding to heterotetrameric complexes of two type I and two type II serine–threonine kinase receptors. Interestingly, the number of ligands in the family (>40, including heterodimers) greatly exceeds the number of available type I (7 total in mammals) and type II receptors (5 total). Ligands therefore bind receptors in a promiscuous fashion. Individual receptor subtypes interact with multiple ligands and a given ligand can bind different receptor combinations depending on the cellular context [4–6]. As a result, both the expression and relative affinities of different receptors (including co-receptors) determine cellular responses to the available ligands.

In pituitary gonadotrope cells, activins classically induce follicle-stimulating hormone (FSH) synthesis by binding to the activin type II receptor ACVR2 [7]. The activin:ACVR2 complex then recruits a type I receptor, usually ACVR1B (or activin receptor-like kinase 4, ALK4), which in turn propagates intracellular signaling *via* recruitment and activation of SMAD2 and SMAD3. Phosphorylated SMAD2/3 then associates with SMAD4 and the forkhead transcription factor FOXL2 to stimulate FSHβ subunit (*Fshb*) transcription, the rate-limiting step in FSH production [8–15].

Other members of the TGFβ superfamily, specifically the bone morphogenetic proteins or BMPs (BMP2, BMP4, BMP6, BMP7, and BMP15)

Abbreviations: TGFβ, transforming growth factor β; BMP, bone morphogenetic protein; BMPR2, BMP type II receptor; ACVR2, activin type II receptor; ECD, extracellular domain; AMHR2, anti-Müllerian hormone type II receptor; TGFBR2, TGFβ type II receptor; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; ALK, activin receptor-like kinase; FST, follistatin; SPR, surface plasmon resonance; RU, resonance unit or response unit; MBP, maltose-binding protein.

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can also modulate *Fshb* expression [16–21]. BMP2, for example, signals *via* the BMP type II receptor (BMPR2) and BMP type IA receptor (ALK3) in gonadotrope-like cells, to potentiate activin effects on *Fshb* transcription [16]. Though BMPR2 was initially identified as a BMP receptor, it has since been shown to bind other ligands as well, such as growth and differentiation factors and inhibins [22,23].

More recent data support the idea that activins also interact with the BMPR2 extracellular domain (ECD) [24,25]. Further, activin is a conformationally flexible molecule, as indicated by the different orientations it can adopt in complex with ACVR2B [26,27]. This flexibility might permit binding to other type II receptors, such as BMPR2. BMPR2 also shows conformational flexibility in its ectodomain, which could in turn facilitate binding by different ligands [28]. Finally, both BMPs and activins, in addition to sharing type II receptors (ACVR2 and ACVR2B), bind follistatin (FST), a soluble binding protein [29,30]. These data suggest that activins and BMPs share structural characteristics that might enable their binding to common receptors, although presumably (and indeed demonstrably) with different affinities.

We hypothesized that activin A might bind and signal *via* BMPR2. As activins classically regulate FSH synthesis, we used LβT2 cells, an immortalized murine gonadotrope-like cell line, as our model system to address this hypothesis. These cells endogenously express both activin receptors and BMPR2 [18], and remain the standard homologous cell line for analysis of *Fshb* expression (*e.g.*, [8,9,11,12,18,31–33]). Our results show that activin A can bind and signal *via* BMPR2 to regulate *Fshb* transcription in these cells.

2. Materials and methods

2.1. Reagents

Recombinant human ACVR2 (340-R2/CF), BMPR2 (811-BR), and TGFBR2 (341-BR) extracellular domain (ECD)-Fc chimeras, as well as human recombinant activin A, BMP2, and TGFβ1 were purchased from R&D Systems (Minneapolis, MN, USA). Enhanced Chemiluminescence (ECL) Plus reagent and $Na^{125}I$ were purchased from Perkin-Elmer (Waltham, MA, USA). Eagle's minimum essential media (EMEM), 100× MEM-non-essential amino acids, 1× Dulbecco's modified phosphate buffered-saline (D-PBS) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, L-glutamine (with or without sodium pyruvate) were from Wisent (St. Bruno, Québec, Canada). DMEM/F12 with 2.5 mM L-glutamine, 15 mM HEPES buffer and 1.2 g/l sodium bicarbonate were purchased from HyClone Laboratories (South Logan, UT, USA). Fetal bovine serum, TRIzol reagent, *Taq* Platinum polymerase, anti-beta-tubulin (cat. #322600), Lipofectamine/Plus and Lipofectamine 2000 were purchased from Invitrogen Canada (Burlington, Ontario, Canada). N-acetyltyrosine, urea, leupeptin, bovine serum albumin (BSA), chloramine T, potassium iodide, trichloroacetic acid (TCA), phenylmethylsulfonylfluoride (PMSF), polyethylenimine (PEI), SB431542, EZview red anti-c-HA affinity gel beads (#E6779), HA peptide (#I2149), mouse monoclonal anti-HA (#H9658), and mouse monoclonal anti-myc (#M5546) antibodies were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Anti-BMPR2 antibody (#612292) was purchased from BD Biosciences (Mississauga, Ontario, Canada). The anti-GAPDH (AM4300) antibody was purchased from Ambion (Austin, TX, USA) and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA). The Nucleospin kit was from Macherey-Nagel (Düren, Germany) and the Lab-Tek II chamber slides from Nunc (Rochester, NY, USA). MMLV-RT, random hexamer primers, RQ1 DNAse, RNAsin, dNTPs and pGEM-T Easy Vector System (cat. #A3610) were purchased from Promega (Madison, WI, USA). Bis-sulfosuccinimidyl-suberate (BS^3) was from Pierce-Thermo Scientific (Rockford, IL, USA), while G-25 sephadex PD-10 columns, G50 columns, and SPR materials were purchased from GE Healthcare (Piscataway, NJ, USA). Protein-grade detergents (Triton X-100, Empigen) were from Anatrace (Maumee, OH, USA) and Pierce Gentle Elution buffer was from Thermo Scientific (Ottawa, ON, Canada). Protease inhibitor tablets (Complete Mini) were purchased from Roche Applied Science (Laval, QC, Canada). D-luciferin potassium salt was obtained from BD Pharmingen (Mississauga, Ontario, Canada). Oligonucleotides were synthesized by IDT (Coralville, IA, USA). Short-interfering (si) RNAs were acquired from Dharmacon (Lafayette, CO, USA): Control (cat. #D-001210-05), *Acvr2* (cat. #D-040676-01), *Acvr2b* (cat. #D-040629-02) and *Bmpr2* (cat. #D-040599-01, -02, -03, and -04). Another set of *Bmpr2* siRNAs was purchased from Qiagen (cat. #SI00930027, SI00930034, SI00930041, SI00930048) along with a negative control siRNA (cat. #1027280).

2.2. Constructs

To clone the murine BMPR2 long isoform (BMPR2-L), 1 μg of LβT2 cell total RNA was reverse transcribed with MMLV-RT, random hexamer primers, and dNTPs as described previously [8]. The cDNA was subject to PCR using *Taq* Platinum polymerase and the following primer set: forward 5′-AGGGATGACTTCCTCGCTGC and reverse 5′-CATCTCACAGACAATTCATTCCT. The PCR reaction conditions were: 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 s, 48 °C for 30 s, 68 °C for 3 min and a final extension step at 68 °C for 10 min. The resulting amplicon was gel-purified (Nucleospin) and TA cloned (pGEM-T Easy Vector System). Recombinant clones were identified by blue–white screening and plasmids were purified (QIAprep, Qiagen). The presence of the insert was verified by restriction digests and bidirectional DNA sequencing (Genome Québec). BMPR2-L was then subcloned into pcDNA3.0 with an in-frame C-terminal HA tag. Finally, we used the QuikChange mutagenesis kit (Stratagene) to correct miss-sense mutations introduced during the initial PCR amplification (reference sequence: accession number NM007561.3). Human BMPR2 short isoform (hBMPR2-S) and human TGFBR2 (generous gifts from Dr. J. Massague, Memorial Sloan Kettering Cancer Center, NY, USA), as well as human AMHR2-myc (provided by Dr. J. Teixeira, Massachusetts General Hospital, MA, USA), rat ACVR2-Flag and rat ACVR2B-Flag (generous gifts from Dr. T. Woodruff, Northwestern University, Chicago, IL, USA), were subcloned into pcDNA3.0 to introduce the C-terminal HA tag. Human BMPR2-S was also subcloned into the pGFP2-N1 vector (Perkin-Elmer) using the restriction sites *Hind*III and *BamH*I to generate a BMPR2-S-GFP2 fusion protein expressing vector. Human BMPR2-ΔC-tail and human BMPR2-K230R were generated by site-directed mutagenesis of the untagged human BMPR2-S construct, by changing residue 177 from glycine to a stop codon or converting the lysine in position 230 into arginine, respectively (see Supplementary Table S1 for primers). Human BMPR2-S-HA and BMPR2-S-GFP2 mutants were also generated using QuikChange mutagenesis with primers indicated in Supplementary Table S1. Sequences of all constructs were verified by bidirectional sequencing. The human ALK3Q233D expression vector was provided by Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan). The 3TP-luc reporter was also a gift of Dr. Massague. The ALK3-myc [16] expression vector as well as −1990/+1 murine *Fshb*-luc [8], −846/+1 murine *Fshb*-luc [34], −3740/+24 murine *Id3*-luc, [35] and $CAGA_{12}$-luc [36] promoter-reporters were described previously.

2.3. Cell lines

Immortalized murine gonadotrope-like LβT2 cells (provided by Dr. P. Mellon, University of California, San Diego, CA, USA) were maintained in 10% FBS/DMEM-high glucose with sodium pyruvate. Human embryonic kidney (HEK) 293 cells (Invitrogen) were grown in 10% FBS/DMEM-high glucose without sodium pyruvate. Chinese hamster ovary (CHO) cells (from Dr. P. Morris, The Population Council, New York, NY, USA) were cultured in 10%-DMEM/F12 media. L17 cells (from Dr. J. Massague) were grown in EMEM supplemented with 10% FBS and 1× non-essential amino acids.