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SMAD3 and EGR1 physically and functionally interact in promoter-specific fashion

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ABSTRACT

Gonadotropin-releasing hormone (GNRH1) stimulates luteinizing hormone β subunit (*LHB*/*Lhb*) transcription. The transforming growth factor β superfamily ligand activin A partially inhibits this effect on the human *LHB* promoter while potentiating GNRH1-induction of the murine *Lhb* gene. Here, we investigated the mechanisms underlying the species-specific modulation of the GNRH1 response by activin signalling. GNRH1 stimulates *LHB*/*Lhb* transcription via induction of early-growth response 1 (EGR1), which binds to the proximal promoter of both species. Activin A decreased GNRH1-induced recruitment of EGR1 to the human, but not murine, promoter. We hypothesized that the activin A signalling protein, SMAD3, might play a role in this system. Indeed, we observed both physical and functional interactions between SMAD3 and EGR1. The two proteins interacted via the SMAD3 MH2 domain and the EGR1 DNA-binding domain. Analogous to the species-specific activin A effect on the GNRH1 response, SMAD3 over-expression partially inhibited EGR1-induction of the human promoter, while potentiating EGR1-induced murine *Lhb* promoter activity. The proximal murine *Lhb* promoter contains three minimal SMAD-binding elements (SBEs) that are absent from human *LHB*. Introduction of the SBEs into the human promoter converted SMAD3 from an inhibitor to a stimulator of EGR1-induced transcription. The converse was observed when the SBEs in the murine promoter were replaced by the corresponding human sequences. Together, our results suggest a model in which activin A inhibits GNRH1-induction of human *LHB* transcription via an interaction between SMAD3 and EGR1 that inhibits the latter's recruitment to the proximal promoter. In contrast, in mouse, the presence of SBEs in the promoter allows SMAD3 and EGR1 to function synergistically to regulate *Lhb* transcription. The basis for their functional cooperativity is not completely clear, but may involve enhancement of EGR1's physical interaction with other important co-factors, including *paired*-like homeodomain transcription factor 1 (PITX1).

1. Introduction

Activins play essential roles in the control of reproductive and other biological processes [1–3]. Like other transforming growth factor β (TGFβ) superfamily ligands, activins signal through hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors [4]. In the canonical activin signalling cascade, type II receptors phosphorylate and activate type I receptors, which then phosphorylate the receptor-regulated SMADs (R-SMADs), SMAD2 and SMAD3. The R-SMADs then homo- and hetero-oligomerize with the co-SMAD, SMAD4, and accumulate in the nucleus where they regulate gene transcription through DNA binding and protein–protein interactions [5–8]. In many instances, SMAD proteins and interacting factors bind adjacent promoter elements to activate genetranscription (e.g. [9,10]). In some contexts, this type of interaction leads to the recruitment of co-repressor complexes and, hence, transcriptional repression (e.g., [11]). SMAD proteins can also repress transcription by interfering with the recruitment of transcription factors to their target promoters (e.g., [12]).

One important site of activin action is the pituitary gonadotrope cell [13–16]. In these cells, activins regulate the transcription of several cell-specific genes, including the follicle-stimulating hormone (FSH) and to a lesser extent luteinizing hormone (LH) β subunits [17–22]. Expression of the *FSHB*/*Fshb* and *LHB*/*Lhb* genes is rate-limiting in the synthesis of the mature dimeric glycoprotein hormones. The primary stimulus for *LHB*/*Lhb* expression is the hypothalamic decapeptide gonadotropin-releasing hormone (GNRH1). GNRH1 induces *LHB*/*Lhb* transcription via the immediate-early gene, early-growth response 1 (EGR1) [23–26]. *In vivo* studies clearly demonstrate the necessity for EGR1 in LH synthesis and fertility [24,25]. EGR1 binds to and activates the proximal *LHB*/*Lhb* promoter in cooperation with other transcription factors, such as *paired*-like homeodomain transcription factor 1 (PITX1) (reviewed in [27]).

Previously, it was demonstrated that GNRH1 and activin A synergistically activate the rat *Lhb* promoter [27]. In contrast, we recently reported that activin A partially inhibits GNRH1-stimulated activation of the human *LHB* promoter [28]. Here, we examined the mechanism underlying species-specific modulation of GNRH1-

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stimulated *LHB*/*Lhb* promoter activity by activins. The data suggest that functional and physical interactions between SMAD3 and EGR1 may underlie activin A modulation of GNRH1 signalling to the *LHB*/*Lhb* promoter.

2. Materials and methods

2.1. Reagents

DMEM with 4.5 g/l glucose and L-glutamine, with or without sodium pyruvate, was from Wisent (St-Bruno, Quebec, Canada). Lipofectamine, Plus reagent, Lipofectamine 2000, gentamycin, SYBR green quantitative PCR master mix, and fetal bovine serum were obtained from Invitrogen (Burlington, Ontario, Canada). Anti-FLAG antibody (F7425), EZview Red M2 FLAG affinity beads, FLAG peptide and chemicals were from Sigma (St. Louis, MO). Taq polymerase, T4 DNA ligase, restriction endonucleases, deoxynucleotide triphosphates and 5× Passive Lysis Buffer (PLB) were purchased from Promega (Madison, WI). ECL-plus reagent and protein markers were from GE Healthcare (Piscataway, NJ). Protease inhibitor tablets (Complete-Mini) were from Roche (Indianapolis, IN). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Anti-SMAD2/3 antibody (07-408) and normal rabbit IgG (12-370) were from Millipore (Billerica, MA), anti-SMAD3 (51-1500) was from Invitrogen (Burlington, Ontario, Canada), anti-phospho-SMAD2 (3101) was from Cell Signalling (Danvers, MA), anti-nucleoporin p62 (610498) was from BD Biosciences (San Jose, CA) and anti-calnexin (SPA-860) was from StressGen (Assay Designs, Ann Arbor, MI). Anti-EGR1 C-19 antibody (sc-189) and Protein A/G PLUS-Agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Constructs

The murine −232/+5 *Lhb* luciferase reporter was produced by PCR amplification of murine genomic DNA and ligated in pA3-luc, as described earlier for the human −196/+9 *LHB*-luc reporter [28]. Mutant promoter-reporter constructs were generated using the QuikChange system (Stratagene). Murine EGR1 (NGFIA) in pJDM464 was a generous gift from Dr. Jeffrey Milbrandt (Washington University School of Medicine, St Louis, MO). Full-length FLAG-tagged EGR1 in pcDNA3 was described earlier [23]. To generate FLAG-tagged truncated EGR1 constructs, the regions corresponding to previously-delineated functional domains [29] were amplified by PCR, using the full-length murine EGR1 as template. The amplicons were cloned in-frame downstream of a FLAG tag coding sequence in pcDNA3. Full-length murine SMAD2 and SMAD3 expression vectors were described previously [18], and full-length human FLAG-SMAD3 and FLAG-SMAD2 in pCAGGS were gifts from Dr. Elizabeth Robertson (Oxford University, UK). FLAG-tagged SMAD3 sub-domains were obtained from Addgene (Cambridge, MA), and are described in detail in [30]. Primer sequences are available upon request. All the constructs were verified by sequencing (Genewiz, South Plainfield, NJ, or Genome-Quebec, Montreal, Canada).

2.3. Cell culture, transfections and reporter assay

LβT2 cells, a gift from Dr. Pamela Mellon (University of California, San Diego, CA), were cultured as previously described [18]. Briefly, for reporter experiments, cells were seeded in 48-well plates, and transfected with 225 ng reporter per well, along with the indicated amount of expression plasmid DNA using Lipofectamine 2000. The next day, transfection media was replaced with serum-free media, and cells were cultured overnight before ligand treatment as indicated. HEK293 cells (a gift from Dr. Terry Hébert, McGill University, Montréal, Canada) were cultured in DMEM without sodium pyruvate, supplemented with 10% FBS. Cells were seeded in 48-wells plate at a density of 30,000 cells/well, and transfected the next day as described for the LβT2 cells. Twenty-four hours later, transfection media were replaced with serum-free media, and cell lysates were harvested the next day. Total DNA transfected was balanced across each condition using the appropriate empty vector. Whole-cell lysates were assayed for luciferase activity as previously described [28]. CHO cells (gift from Dr. Patricia Morris, Population Council, New York, NY) were cultured in DMEM/F12 supplemented with 10% FBS.

2.4. Co-immunoprecipitation analyses and western blotting

Lysates from CHO or LβT2 cells were cultured, transfected, and processed for co-IP using anti-FLAG M2 agarose affinity beads as previously described [31]. Nuclear and cytoplasmic extracts were prepared following published methods [32]. Western blotting was performed as described in [18].

2.5. Chromatin immunoprecipitation

LβT2 cells in 10-cm dishes were transfected with the human −196/+9 *LHB*-luc construct, and treated for 2 h with 10^{-7} M GNRH1 and/or 25 ng/ml activin A. After treatment, formaldehyde was added to a final concentration of 1%, and crosslinking performed for 10 min at room temperature. The crosslinking reaction was quenched with 125 mM glycine for 5 min. Cells were then lysed in 1 ml lysis buffer [1% SDS, 1 mM EDTA, 50 nM Tris–HCl (pH 8), protease inhibitors]. Half of the lysate was sonicated with six 5-s pulses at power 0.5 using a Misonix Sonicator 3000 (Misonix, Farmingdale, NY). The sonicated chromatin was spun for 10 min at 13,000 rpm to pellet cellular debris. Two hundred microliters of sonicated chromatin was added to 1800 µl dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8, 16.7 mM NaCl, protease inhibitors) and pre-cleared for 30 min at 4 °C with 75 µl protein A/G-agarose bead slurry (1:3 volume of protein A/G-agarose beads in a solution containing 10 mM Tris, 1 mM EDTA, 0.1% BSA, 0.27 µg/µl salmon sperm DNA). Beads were pelleted at 3000×*g* for 5 min at 4 °C. One-twentieth of the volume of pre-cleared chromatin was removed and kept as "input" chromatin. The remaining chromatin was divided in two, and each half incubated overnight with 60 µl of the protein A/G-agarose beads slurry (see above) and 5 µg anti-EGR1 antibody or normal rabbit IgG. The next day, beads were sequentially washed with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 nM Tris pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 nM Tris pH 8, 500 mM NaCl), LiCl buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8), and twice in TE buffer (10 mM Tris pH 8, 1 mM EDTA). Each wash was performed for 5 min at 4 °C, followed by a 30-s spin at 3000×g. DNA was eluted for 15 min at room temperature in 480 µl elution buffer (1 M $NaHCO_3$, 1% SDS). NaCl was added to a final concentration of 0.3 M and protein:DNA complexes were reverse-cross-linked overnight at 65 °C. The next day, samples were incubated for 30 min at 37 °C with 20 ng/µl RNase A. Tris–HCl (pH 6.8) and EDTA were added to final concentrations of 400 mM and 100 mM, respectively, and samples were incubated at 45 °C for 2 h with 20 ng/µl proteinase K. DNA was extracted with phenol:chloroform and precipitated with ice-cold ethanol for 30 min in the presence of 30 pg/µl tRNA at −80 °C. DNA was pelleted at 13,000 rpm at 4 °C for 15 min, washed with 75% ethanol, dried, and dissolved in clean water. One-thirtieth of each immunoprecipitation product and 1/15,000 of each input sample was analyzed in triplicate by real-time quantitative PCR with SYBR Green qPCR Master Mix using a Corbett Rotor-Gene 6000 instrument. Primer sequences are available upon request. For quantification, the calculated chromatin concentration (determined with the relative standard curve method)