A Smad-binding Element in Intron 1 Participates in Activin-dependent Regulation of the Follistatin Gene

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Amy L. Blount, Joan M. Vaughan, Wylie W. Vale1, and Louise M. Bilezikjian2
From the Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, California 92037

Follistatins exert critical autocrine or paracrine control in many tissues by binding and bio-neutralizing activin and several other transforming growth factor-β ligands. In the pituitary, activin acts locally to induce follistatin expression and thus modulate its own actions. This local feedback loop safeguards against excessive activin signaling and maintains the necessary balance of activin and follistatin tone. To better understand the mechanisms underlying the activation of follistatin by activin A, follistatin transcription was evaluated in gonadotrope-derived αT3-1 cells. Transient transfection experiments established that follistatin-luciferase plasmids that incorporate up to 2.86 kb of the upstream region of the rat follistatin gene are not induced by activin A in αT3-1 cells. On the other hand, plasmids that incorporate intron 1 are responsive to activin A and induced by a constitutively active form of ALK4. These experiments ultimately identified a conserved Smad-binding element (SBE1) in intron 1, between +1791 and +1795. In these experiments, activin A in αT3-1 cells treated with activin A, SBE1 preferentially recruits Smad3, but not Smad2, and mediates Smad3-dependent activation of follistatin transcription. shRNA knockdown of endogenous Smad3 in these cells compromises SBE1-mediated transcription in response to activin A and interferes with its ability to positively regulate follistatin mRNA levels. The findings of the current work illustrate the critical role of intron 1 of the follistatin gene in mediating Smad-dependent effects of activin and regulating the expression level of this gene in some cell types, such as pituitary cells of gonadotrope lineage.

Activins are members of the evolutionarily conserved transforming growth factor-β (TGF-β)1 superfamily of factors implicated in the control of a wide array of cellular processes of embryonic and adult tissues (1–3). Activin A and activin B are homodimers of inhibin βA and βB subunits, respectively, with established roles in the control of many cell types including those throughout the reproductive axis (1, 4–6). The inhibin βA and βB subunits are both expressed in the pituitary and activin B arising from gonadotropes is a critical component of the network of autocrine or paracrine factors of this tissue (7). By exerting control on FSHβ expression, activin B is hypothesized to locally provide a positive signal for the differential production of FSH over LH and to thereby facilitate the cyclic fluctuations of these hormones during the estrus cycle (7, 8). The actions of activins are strictly controlled by the concerted actions and the preferential usage of several extracellular modulators (9, 10). Of these, inhibin and follistatin have established roles in the regulation of pituitary gonadotropes (7).

Follistatins are cysteine-rich glycoproteins that bind and bio-neutralize activin with high affinity at a 2:1 molar ratio (11–13). They also bind and inactivate myostatin and some bone morphogenetic proteins with varying degrees of affinity (14). Structural studies of the follistatin-activin complex have elucidated the basis for this interaction and provided clues about the determinants of follistatin binding to other ligands (15, 16). The follistatin gene comprising 6 exons produces two alternatively spliced mRNA transcripts and the corresponding proteins of 315 or 288 amino acids (FS315 or FS288) (17, 18). These isoforms, FS315 and FS288, are differentially expressed in most tissues (19, 20). The FS315 form is the predominant circulating form, whereas the shorter FS288 form lacking the C-terminal tail associates tightly with heparan sulfate chains of cell surface proteoglycans and is presumed to be the form that acts locally to modulate activin signaling (2, 21–23). This local function of follistatin is, in turn, controlled by the actions of activin. In cultured rat anterior pituitary cells, activin increases steady-state follistatin mRNA and secreted protein levels (24–28). Given that inhibin antagonizes the activin-induced rise in pituitary follistatin, this pinpoints gonadotropes as the primary targets of this action of activin (28, 29).

Activin signals by sequentially binding to activin-specific Type II (ActRII or ActRIIB) then Type I (ALK4) serine/threonine kinase receptors to form a multimeric complex (3, 30, 31). In this complex, the Type II receptors trans-phosphorylate Type I (ALK4) and allow it to transiently interact and phosphorylate the downstream signaling proteins, Smad2 and Smad3, at their C-terminal SSXS motifs (31–33). The phosphorylated forms of Smad2 and Smad3 in turn translocate to the nucleus where they bind to DNA targets and regulate transcription in association with the common Smad4/DPC4 and other partners (34).

The mechanism by which activin induces follistatin expression is not well characterized. A luciferase reporter construct that incorporates the −757/+136 fragment of the rat follistatin...
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Experimental Procedures

Primary Cells and Cell Lines—Primary rat anterior pituitary (RAP) cells were prepared by collagenase-mediated digestion of anterior pituitaries obtained from male Sprague-Dawley rats (180–200 g) as previously described (24). The dispersed RAP cells were seeded on tissue culture plates and maintained at 7.5% CO₂ in a humidified 37 °C incubator in a specially formulated medium (designated βP) containing appropriate growth factors and 2% fetal bovine serum (FBS) (24). The cells were allowed to recover for at least 3 days before initiating experiments. The mouse gonadotrope-derived αT3-1 (42) and the human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 2 mM glutamine.

Analysis of Follistatin Transcript Levels by RNase Protection Assays and Real-time PCR—On the 4th day following dispersion, RAP cells (10⁷ per 10-cm tissue culture dish) were washed and equilibrated overnight in with 0.2% FBS in βP medium, then washed again and treated in fresh medium with activin A or TGF-β (40, 41). The present study was undertaken to further evaluate the mechanism involved in the transcriptional activation of the follistatin gene by activin to identify activin-responsive regulatory elements. The mouse αT3-1 gonadotrope cell line, which expresses endogenous follistatin, was used as a cellular model. The results indicate that activin induction of the follistatin gene is mediated by a conserved Smad-binding element that localizes to the first intron.
incubating them for 6 h with a mix of the Superfect Transfection Reagent (Qiagen; Hilden, Germany), 0.6 μg/well of luciferase reporter plasmid and 0.2 μg/well cytomegalovirus (CMV)-β-galactosidase (β-Gal) plasmid as an internal control. Where indicated, varying amounts of expression plasmids encoding Myc-tagged Smads, caALK4, or empty vector were co-transfected along with the reporters. At the end of the 6-h transfection period, the cells were washed and treated with vehicle or activin A in Dulbecco’s modified Eagle’s medium supplemented with 2% FBS and 2 mM glutamine. The cells were harvested 15 h later in lysis buffer (1% Triton X-100, 25 mM glycyglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT). Luciferase reporter activity was measured using d-luciferin luciferase substrate (Biosynth, Naperville, IL) with a LuminoMark microplate luminometer (Bio-Rad) or a Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany) and normalized to that of CMV-β-Gal. Reported data correspond to luciferase/β-Gal ratios of each plasmid relative to the activity of the pGL2 basic vector.

Chromatin Immunoprecipitation (ChIP)—The method used for ChIP analysis was essentially as described previously (43). Activin A or vehicle-treated αT3-1 cells were cross-linked with 1% formaldehyde for 15 min at room temperature. The cells were lysed by incubating them for 10–15 min on ice in lysis buffer (25 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 10 mM KC1, 0.1% Nonidet P40, 1 mM DTT, and protease inhibitors). The nuclear fraction that was recovered by centrifugation (5 min at 5000 × g) was resuspended in ChIP buffer (50 mM HEPES, pH 7.8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors) and sonicated on ice (Misonix XL200 ultrasonic cell disruptor) to achieve an average chromatin length of 500–1000 bp. The sonicated samples were precleared by incubation with protein A-Sepharose in the presence of 8 μg/ml salmon sperm DNA, 0.3% normal rabbit serum, and 0.05% bovine serum albumin followed by centrifugation. The material recovered from the equivalent of ~10⁷ αT3-1 cells was incubated overnight at 4 °C with 5 μl of either normal rabbit IgG, anti-hSmad2/3, or anti-hSmad2/3 preabsorbed with the peptide antigen along with protein A-Sepharose. The protein A-purified rabbit anti-hSmad2/3 used for these experiments is directed against a peptide within the linker of hSmad2 (amino acids 199–215), which is conserved in hSmad3. The protein A-Sepharose beads were washed sequentially once with ChIP buffer, twice with ChIP buffer containing 0.5 mM NaCl, once with 0.25 M LiCl buffer (20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA), and finally, twice with TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The specifically bound complexes were eluted from the protein A-Sepharose beads by two 15-min incubations at 65 °C with TE elution buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% SDS). The immunoprecipitated complexes and the starting material (input) were incubated overnight at 65 °C to reverse cross-linking, then treated with proteinase K and purified using QIAquick Spin Columns (Qiagen, Hilden, Germany). The DNA samples were recovered in 50 μl of 10 mM Tris-HCl, pH 8.5, and analyzed by semiquantitative PCR using primers that amplify a fragment of 185 bp overlapping the SBE1 site within intron 1 of the endogenous mouse follistatin gene (forward: 5'-GTCGCTGCAGTTATGAAATGG and reverse: 5'-AAAGGGGAGAGTTGGGAAGGAC). The amplified fragment was then resolved on a 2% agarose gel and analyzed by ethidium bromide staining. Alternatively, DNA from 1–2 μl of each sample was quantified by real-time PCR using the SYBR GREEN PCR Master Mix and the ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA). The fragment containing the SBE1 site was amplified using primers flanking the site (forward: 5’-AACAGTCTAGTAAAGTGCATTGCAAGCT and reverse: 5’-TGCGCCCCACCCCATAT) to verify amplification. A primer set that amplifies a fragment ~5-kb upstream of the transcription start site of the mouse follistatin gene (forward: 5’-AGATAGAGATCCACACACAGAACA and reverse: 5’-GGATGGACTTGGGTGGTATCTGTA) or a fragment of the mouse β-actin gene (forward: 5’-TTCCCTTCCACAGGTTGTA and reverse: 5’-ACATAGAGTCTCTTCTGACCCATT) were used as internal controls. Primer pairs flanking the upstream putative Smad3 site at −1604 (forward: 5’-CGGCTGTATTTCCGGATCTATT and reverse: 5’-ACTCGAGGATATGCTGATTCCATT and reverse: 5’-CCCTCGGGCTCCACAACT) were used to amplify the corresponding fragments.

Oligonucleotide Precipitation Assays—For these experiments, a lentiviral delivery system was used to facilitate the expression of Myc-tagged Smads in αT3-1. The N-terminally Myc-tagged hSmad2, -3, or -4 cDNAs were subcloned upstream of an IRES GFP marker in the pCSC-SP-PW-IRE/GFP lentiviral transfer vector (generously provided by Dr. Inder Verma, Salk Institute, La Jolla, CA). The GFP-expressing pCSC-SP-PW-IRE/GFP empty vector was used as control. Recombinant lentiviruses were produced by co-transfecting HEK293T cells with the Smad expression or control transfer plasmid and three additional plasmids required for packaging (pMDL, pRev, and pVSVG) using polyethylenimine as the transfection reagent, as described (44). The supernatants containing the viral particles were collected 48 h after transfection, filtered through a 0.45-μm filter, and concentrated by ultracentrifugation for 2.5 h at 50,000 × g. Relative titers were assessed by monitoring the percentage of GFP-positive HEK293T cells infected with serial dilutions of the viral preparations. For oligonucleotide precipitation experiments, αT3-1 cells (5 × 10⁶/10 cm dish) were infected in the presence of 8 μg/ml polybrene (Sigma) with lentiviral vectors to express only GFP as a control or combinations of Myc-Smad2 and -4 or Myc-Smad3 and -4. The amount of virus necessary to achieve >90% GFP-positive αT3-1 cells was predetermined by performing serial dilutions of equivalent titers of the viral preparations. Protein expression was allowed to progress for 5 days at which time the cells were replated into four 10-cm dishes and allowed to grow for two more days. The cells were supplemented with fresh medium and treated for 30 min with 1 nM activin A or vehicle in duplicate. Lysates of αT3-1 cells were prepared by brief sonication in lysis buffer (25 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 10% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 100 mM NaCl, 5 mM NaF, 1 mM Na₃P₂O₇, 1 mM DTT, and protease inhibitors) folowed by a 10-min centrifugation at 12,000 × g at 4 °C. The supernatant obtained from each 10-cm dish (850 μg) was incu-
bated for 2 h at 4 °C with 1 μg of biotinylated double-stranded oligonucleotides, precoupled to streptavidin-agarose beads (Pierce), in the presence of 8 μg of poly(dI-dC) (Sigma). The agarose beads were washed three times by centrifugation, and specifically bound proteins were recovered and subjected to Western analysis. The samples were resolved under reducing conditions using 10% NuPAGE SDS gels (Invitrogen) and MOPS as the running buffer then transferred to nitrocellulose membranes. After blocking the membranes with 5% BLOTTO (Pierce), Myc-Smads were detected using an anti-Myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ). Immune complexes were then visualized with SuperSignal West Pico chemiluminescence substrate (Pierce). The experiments were performed using biotinylated wild type (forward: 5′-CAAGCTTCG-CACGTTGTGTCTCGGGTGTAACTGACATTGTGATCTAGCTAAGGCGAGCGCTGCTGCTC; reverse: 5′-biotin-GAGCAGCAGCCGCTGCGCTAGCCATATCATACGT-TACAAGCTG-ACAGGCCTTTACAGCTTCT (S2#1) 5′-GATTGAACTTCATCTGAAT (S2#4). The shRNA cassettes were generated by PCR amplification from pSuper (Oligoengine, Seattle, WA) using a forward T3 primer and reverse primers (5′-CAAGCTTGCTAGGCCTAGCCTGCTGCTC; antisense target) TCTCTTGAA (antisense target) CACGTGTTGTGTCTGGGTCACTGGTAACTGACATTGAGCAGCAGCCGCTGCGCTAGCCATATCATACGT-TACAAGCTG (S3#2), and 5′-GATTGAACTTCATCTGAAT (S3#3), and 5′-biotin-GAGCAGCAGCCGCTGCGCTAGCCATATCATACGT-TACAAGCTG (S3#4). The shRNA cassettes were inserted into the compatible NheI site in the 3′-LTR of the lentiviral IRES vector, p156RRLSINpptCMVGFPPRE, that incorporates CMV-driven GFP as a marker (generously provided by Dr. Inder Verma, Salk Institute). Viral particles were prepared using HEK293T cells, in the presence of 80 μg/ml polybrene as described above. Fresh medium was introduced 24 h later, and the cells were cultured for 3 additional days. The cells were subsequently processed for transient transfection experiments, as described above, with rFS(0.3ex45)-luc as the reporter. Alternatively, the cells were supplemented with fresh medium, then treated with either vehicle or activin A for 2 h. These cells were then processed for follistatin mRNA determination. Total RNA was extracted using the RNeasy Micro kit (Qiagen) and reverse-transcribed using SuperScript II (Invitrogen). To quantify endogenous mouse mRNA levels, real time PCR was performed on the ABI 7900HT Fast Real-Time PCR System using Power SYBR Green Master Mix (Perkin-Elmer Applied Biosystems). Follistatin transcript levels were quantified using the ΔΔCt, relative quantification method (48) (forward primer: 5′-CCCCCA-CTGCATCCCTGTGTA and reverse primer: 5′-GTGTTGCCG-AGTCCAGTTT) with GAPDH as control (forward primer: 5′-GGAAGGGCTCATGACCACAGT and reverse primer: 5′-CACAGTCTTCTGAGTCAGT). Regressions—Recombinant human activin A was purified from the conditioned medium of stably transfected CHO cells (generously provided by Dr. Wolfgang Fischer, Salk Institute, La Jolla, CA). The human Smad2 and Smad3 cDNAs (provided by Dr. Rik Derynck, University of California at San Francisco) and the human Smad4/DPC4 cDNA (provided by Dr. Scott Kern, Johns Hopkins University School of Medicine, Baltimore, MD) were modified by adding a Myc tag at the N terminus and cloned into the CS2+ expression vector. The expression plasmid for the constitutively active Type I activin receptor (ALK4/T206D) or caALK4 (ALK4) has been described previously (49).

RESULTS

Activin Increases Follistatin Transcript Levels of Primary Rat Anterior Pituitary and αT3-1 Cells—Activin A causes a dramatic increase in follistatin mRNA levels and follistatin secretion from primary cultures of rat anterior pituitary (RAP) cells, as previously reported (24, 28). Several lines of evidence suggest that this reflects the transcriptional activation of the follistatin gene in pituitary gonadotropes (39). To better understand this mechanism and identify a suitable cell line for further studies, activin effects on follistatin mRNA expression were evaluated in greater detail in RAP cells and compared with those of gonadotrope-derived αT3-1 cells. In RAP cells, 1 nM activin A caused a rapid and transient increase in primary follistatin transcript levels accompanied by a delayed slower rise of the mature mRNA form (Fig. 1, panel a). Co-treatment with inhibin A antagonized the activin-induced rise in follistatin mRNA levels suggesting that the observed changes in follistatin transcript levels reflect primarily those occurring in inhibin-responsive
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![Image of the diagram](image)

**FIGURE 1.** Activin A and inhibin A regulate follistatin transcript levels of primary cultures of RAP cells (panels a, b, and c) and αT3-1 (panels d, e, and f) cells. Panels a and d, time-dependent effects of 1 nM activin A; panels b and e, inhibin A (2 nM) antagonism of 2 h of activin A (1 nM) effect; panels c and f, concentration-dependent effects of activin A (1 h). The total amount of RNA extracted from the nuclear or cytosolic fraction of 10^7 cultured RAP cells or 50 μg of total RNA extracted from mouse αT3-1 cells was used for each point. The antisense riboprobe corresponding to the rat follistatin gene was designed to protect a 463 nt of primary transcript corresponding to the portion that spans the junction of exon 3/intron 3 and a 203 nt portion of only exon 3. The mouse antisense riboprobe protects 470 nt of primary transcript spanning exon 3/intron 3 and 190 nt of exon 3. The RNA samples were hybridized with the corresponding riboprobes, as detailed under "Experimental Procedures," resolved on a 5% polyacrylamide, 8 M urea gel and visualized using the PhosphorImager system. The reported mean ± S.E. values are derived from triplicate determinations of representative experiments.

gonadotropes (Fig. 1, panel b). As in RAP cells, gonadotrope-derived αT3-1 cells responded to activin A with a similar rise in primary and mature follistatin transcript levels that was also antagonized by co-treatment with inhibin A (Fig. 1, panels d and e). The effect of activin A on follistatin transcript levels of both αT3-1 and RAP cells was concentration-dependent over the range tested (Fig. 1, panels c and f). Based upon these observations, the αT3-1 cells were used for further evaluation of the follistatin gene.

**Regulatory Elements in the First Intron of the Follistatin Gene Are Responsive to Activin**—To define the mechanism of transcriptional activation of the follistatin gene in pituitary gonadotropes and localize activin-responsive elements, fragments of the rat follistatin gene were cloned upstream of the firefly luciferase reporter in pGL2, and evaluated for inducibility by activin A in αT3-1. First, the region from −2864 to +136 corresponding to the proximal promoter of the rat follistatin gene was cloned into pGL2 (rFS(2.9)-luc) and evaluated. Although this upstream fragment displayed basal activity in αT3-1 cells, it failed to mediate activin A effects (Fig. 2a). Equivalent plasmids that were truncated at the 5′-end to either −752 (data not shown) or −312 (rFS(0.3)-luc) behaved similarly and remained unresponsive to activin A (Fig. 2a). These observations suggested that activin-responsive elements must reside further upstream or downstream. To evaluate the latter possibility, intron 1 (1870 bp) of the rat follistatin gene was cloned at the 3′-end of the −2864/+136 fragment, and the resulting plasmid (rFS(2.9)-luc) was evaluated in αT3-1 cells. Activin A induced the reporter activity of the rFS(2.9)-luc plasmid 8-fold indicating that regulatory elements located in intron 1 mediate this effect (Fig. 2a). The removal of the region upstream of −312 somewhat compromised the magnitude of activin inducibility of rFS(0.3i)-luc relative to rFS(2.9i)-luc (Fig. 2a). Nevertheless, both plasmids were activated in αT3-1 over the same concentration range of activin A (Fig. 2b). These results suggest that regulatory elements between −2864 and −312 do not contribute significantly to establishing sensitivity to activin A. Hence, further analyses were confined to plasmids that contained only 312 bp of the proximal promoter of the rat follistatin gene. Responsiveness to activin A was lost by the removal of a 129-bp fragment (+1784/+1912) toward the 3′-end of intron 1, indicating that this fragment participates in mediating activin A effects (Fig. 2a). Indeed, this +1784/+1912 fragment corresponding to the 3′-end of intron 1 conferred activin A responsiveness to the otherwise unresponsive rFS(0.3i)-luc plasmid (compare the activity of rFS(0.3)-luc to rFS(0.3iex45)-luc (Fig. 2a). The pattern of activin-responsiveness of the various plasmids described thus far was recapitated by co-transfected caALK4 (Fig. 2a). This intronic fragment of the rat follistatin gene, herein referred to as the activin-responsive fragment (ARF), has enhancer-like activity as it mediated activin A effects when two or more copies were placed upstream of the unresponsive rFS(0.3i)-luc or the heterologous plgL2-(SV40)-pro- plasmid (Promega) (Fig. 2c). A series of 3′ truncations within ARF using exonuclease III defined a minimal fragment of intron 1 (from +1784 to +1912) capable of activating transcription in response to 1 nM activin A (data not shown) or co-transfected caALK4 (Fig. 2d). Altogether, these results suggest that ALK4-mediated activin signaling in gonadotropes induces follistatin gene transcription via regulatory elements located within the ARF of intron 1.

**Smad Signaling Activates Follistatin Gene Transcription**—To determine whether the Smad signaling pathway participates in mediating the transcriptional effects of activin on the rat follistatin gene, co-transfection experiments with hSmad2, -3, and -4 expression plasmids were performed in αT3-1 cells. Consistent with the data of Fig. 2, whereas the rFS(2.9i)-luc plasmid that incorporates intron 1 was activated in cells treated with activin A, rFS(2.9i)-luc lacking intron 1 was not (Fig. 3a). Neither hSmad2 nor hSmad4 alone or together had an appreciable effect on either reporter plasmid (Fig. 3a). Co-transfected hSmad3, on the other hand, induced the basal activity of rFS(2.9i)-luc, but not rFS(2.9i)-luc lacking intron 1 (Fig. 3a). The combined actions of hSmad3/4 enhanced the basal and activin-dependent reporter activities of both rFS(2.9)-luc and rFS(2.9i)-luc plasmids without and with intron 1, respectively (Fig. 3a).
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These results illustrate that full activin/Smad-dependent activation of the rat follistatin gene in αT3-1 cells requires elements in intron 1 but that upstream elements may also contribute to this process.

A Smad-binding Element in Intron 1 Mediates Activin Effects on Follistatin Gene Transcription—Examination of the rat follistatin gene with the MatInspector sequence analysis tool (Genomatix Software GmbH, Munich, Germany) (50) identified a putative Smad4 (at −895) and a Smad3 (at −1604) binding site in the 5′-flanking region (Fig. 4a). More importantly, further analysis suggested the presence of a Smad3 binding motif (5′-GTCTGggtca-3′) located at +1791/+1800 of intron 1 (Fig. 4a). Because the data of Fig. 3a supported the potential importance of a downstream Smad-binding element, this intronic Smad3 binding site, designated SBE1, was subjected to further evaluation. To test its contribution in mediating Smad-dependent transcription of rat follistatin, the SBE1 site of the rFS(0.3ex45)-luc reporter was mutated by introducing 3-nucleotide substitutions within the putative core Smad binding site (Fig. 4a), and this mutant plasmid was then tested in αT3-1 cells. By contrast to the wild-type rFS(0.3ex45)-luc plasmid, the corresponding SBE1 mutant plasmid was not induced by activin A nor was it activated by co-transfected hSmad3/4 (Fig. 3b) or caALK4 (Fig. 3c). Collectively, these results suggest that SBE1 has an important role in mediating Smad-dependent transcriptional effects of activin A in αT3-1 cells.

Activin A Induces the Recruitment of Smad3 to the SBE1 Site of Intron 1 of the Follistatin Gene in αT3-1 Cells—Sequence alignment revealed that intron 1 of the rat follistatin gene is 93% identical to the mouse gene and, more importantly, that the SBE1 site is fully conserved in both genes. ChIP assays were performed to evaluate the participation of endogenous Smad signaling and the importance of SBE1 in mediating the transcriptional effects of activin A on the mouse follistatin gene of αT3-1 cells. A purified rabbit polyclonal antibody (anti-Smad2/3) used for these experiments was first validated for its specificity (Fig. 5a). Semiquantitative analysis of samples immunoprecipitated with anti-Smad2/3 but not normal rabbit IgG, yielded an activin A-inducible PCR-amplified fragment of the expected size corresponding to mouse follistatin (Fig. 5b). The primer set used for these analyses amplifies a 185-bp follistatin fragment that spans the entire ARF of intron 1. Further analysis using real-time PCR confirmed that activin A induced a time-dependent (Fig. 5c) and a concentration-dependent (Fig. 5d) recruitment of Smad2/3 to the SBE1 site of the endogenous mouse follistatin gene in αT3-1 cells. Co-treatment with recombinant FS288 bio-neutralized and prevented this action of activin A (Fig. 5e). By contrast to these results, flanking regions corresponding to the putative Smad3 and Smad4 binding sites at positions −1604 and −895, respectively, were not enriched by anti-Smad2/3 (Fig. 5f).
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Because the antibody used in ChIP analyses does not distinguish between Smad2 and Smad3, those experiments could not determine the relative recruitment or the importance of the endogenous proteins in mediating the transcriptional effects of activin A while the plasmid lacking intron 1 (rFS(2.9)-luc) is marginally activated by co-transfected hSmad3/4. (Inset shows a representative Western analysis of co-transfected Smad levels.) b, mutagenesis of the SBE1 site abolishes transcriptional activation of rFS(0.3ex45)-luc in response to 1 nM activin A or co-transfected hSmad3/4. c, mutagenesis of the SBE1 also abolishes responses to caALK4. The ΔT3-1 cells were co-transfected with the indicated luciferase reporter plasmids, CMV-β-Gal, and Myc-tagged hSmad2, -3, or -4 expression vectors as shown. Details of the substitutions within SBE1 to generate mSBE1 are shown in Fig. 4. For these experiments, transiently transfected ΔT3-1 cells were treated with either vehicle or 1 nM activin and processed as described above. Data represent arbitrary light units (L.U.) internally normalized to β-Gal activity and reported relative to pGL2. The results of triplicate determinations of representative experiments are reported as mean ± S.E.

shRNA Knockdown of Smad3 Attenuates Activin-dependent Induction of Endogenous Follistatin mRNA—The experiments described thus far suggest that Smad3 is the preferential mediator of activin A effects on follistatin expression in gonadotropes. To determine if this is indeed the case, an shRNA knockdown strategy was used to evaluate the relative importance of endogenous Smad2 and Smad3 in mediating activin A effects. Four shRNAs targeting each of Smad2 or Smad3 were designed and evaluated in HEK293T cells for their ability to knockdown the expression of the corresponding Smad as compared with the empty vector control. Of the Smad2 targets tested, S2#1 and S2#2 were only marginally effective and reduced Smad2 to -50% of control levels (Fig. 7a). By contrast, Smad2 shRNAs S2#3 and S2#4 reduced Smad2 to levels that were undetectable (Fig. 7a). Of the four Smad3 shRNAs, S3#3 was totally ineffective whereas S3#1, S3#2, and S3#4 significantly reduced the level of Smad3 protein detectable by Western analysis. These effects were specific in that shRNAs targeting Smad2 did not produce detectable changes in Smad3 or Smad4 expression and, conversely, shRNAs targeting Smad3 did not influence Smad2 or Smad4 levels (data not shown). Substantial knockdown was measurable within 48 h of transduction. The shRNA plasmids that were validated to be effective by Western analysis (S2#4 and S3#4) were then tested for their ability to compromise activin-induced rFS(0.3ex45)-luc activity in ΔT3-1 cells infected with lentiviral vectors. The basal
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Activins are key modulators of follistatin expression and many tissues that express and respond to activin often also produce follistatin (2, 7). This close anatomic and functional link establishes a local feedback loop that safeguards against excessive activin signaling and insures that the necessary balance of activin and follistatin tone within a given tissue is maintained. Accordingly, it is presumed that many of the demonstrated actions of follistatins reflect their influence as local “buffers” of the bioactivity of activins and other members of the TGF-β family of ligands (2, 14). In the anterior pituitary, the self-modulating mechanism through which activin induces follistatin production exerts control over activin bioactivity and plays a pivotal role in maintaining FSHβ expression and FSH production from gonadotropes at levels that are physiologically relevant and necessary for normal reproductive function (7, 8). Several lines of evidence also suggest that a balanced activin and follistatin tone is a critical checkpoint for the control of pathogenic mechanisms underlying tumor formation within the pituitary (51, 52). Genetic models support these far-reaching actions of follistatin. The overexpression of follistatin in mice is associated with reduced FSH levels, subfertility arising from gonadal defects as well as abnormalities of the skin and hair formation (53). Mice with a targeted deletion of the follistatin gene, on the other hand, die within hours of birth because of severe gonadal defects as well as abnormalities of the skin and hair (54). Despite the established importance of
follistatin, the mechanisms or signaling pathways that regulate its expression and mediate the effects of activin have not been extensively explored.

To start unraveling the mechanism of activin-induced follistatin expression, the mouse αT3-1 cell line was used as a model of pituitary gonadotropes to evaluate the transcriptional regulation of this gene. The findings of the current study confirm that follistatin is a transcriptional target of activin A in αT3-1 cells. The results further indicate that activin inducibility of follistatin in this cell type is controlled by a Smad-binding element (SBE1) located within a regulatory region of intron 1 (ARF). This intronic ARF displays activin-responsive enhancer activity and mediates activin A effects from either upstream or downstream sites, albeit more weakly than in its native context. The latter is consistent with the possibility that other regulatory regions cooperate with SBE1 and contribute to full transcriptional activation of the follistatin gene.

The results presented here suggest that the SBE1 element identified in the current study mediates Smad3, and to a much lesser extent Smad2 effects and that Smad3 is the preferential downstream mediator of activin in αT3-1 cells. This conclusion is supported both by shRNA knockdown and oligonucleotide pull-down experiments. shRNA-mediated knockdown of Smad3 significantly compromised the ability of activin A to regulate SBE1-dependent transcription and, more importantly, to induce endogenous follistatin mRNA accumulation in αT3-1 cells. Furthermore, oligonucleotide precipitation experiments demonstrated that activin A induces the association of Smad3, but not Smad2, to a biotinylated probe that integrates the wild-type SBE1. This association was probably not facilitated by Smad4 because activin-dependent Smad3 association with the wild-type SBE1 probe was observed regardless of whether Smad4 was co-expressed (Fig. 6) or not (data not shown). Moreover, the observed Smad4 association with the wild-type probe was independent of activin A. These results suggesting that Smad4 does not contribute to the inducible assembly of Smad3 complexes to SBE1 are consistent with reporter assays that failed to reveal Smad4-dependent activation via SBE1.

In regards to Smad2, the failure of the wild-type probe to recruit it could have resulted from the absence of FoxH1 in αT3-1 cells, given that FoxH1 is an obligatory cofactor of Smad2 at some downstream targets of TGF-β ligands (55–57). This was not the case, however, because concurrent expression of FoxH1 and encoding Myc-tagged hSmad2 or -3. Immunoblots that were sequentially probed with a mAb anti-Myc then a mAb anti-actin as an internal loading control were subjected to ECL for visualization of immune complexes. b, concentration-dependent effects of activin A on the activity of the rFS(0.3exo45)-luc reporter plasmid in αT3-1 cells transduced with the empty lentiviral vector as control or vectors encoding Smad2 (S2#4) or Smad3 (S3#4) targeting shRNAs. The results of a representative experiment, performed in triplicate, are shown as the ratio of measured luciferase activity to β-Gal.

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Smad2, with or without Smad4, did not activate activin-responsive plasmids such as rFS(2.9i)-luc, rFS(0.3i)-luc or rFS(0.3ex45)-luc plasmids above baseline (data not shown). Therefore, unlike the intronic enhancers of lim-1, Xnr1 and the intronic left side-specific enhancer of mouse Nodal (58–60), the Smad-binding element in intron 1 of the rat follistatin gene does not assemble FoxH1-Smad2 complexes.

Initially, DNA fragments corresponding to the upstream promoter region of the rat follistatin gene were evaluated for their ability to mediate activin A effects in transiently transfected αT3-1 cells. Primer extension analyses confirmed that both αT3-1 and primary rat anterior pituitary cells preferentially utilize the “α” transcription start site and to a lesser extent the “β” and “γ” sites previously identified from studies of granulosa, P19 and F9 embryonic carcinoma cells (35, 36). An upstream fragment equivalent to −2864 to +136, relative to the transcription start site of the rat follistatin gene, failed to confer activin inducibility onto the basic pGL2 luciferase reporter. The same construct was only weakly (1.5-fold) activated in αT3-1 cells when the Smad signaling pathway was activated directly via co-transfection of a constitutively active form of ALK4 (caALK4). Truncating the −2864/+136 fragment at the 5′-end to −752 (data not shown) or −312 reduced basal reporter activity but did not influence activin responsiveness confirming that elements upstream of the −312 position of the rat follistatin gene do not contain inhibitory sites nor are they sufficient for mediating the transcriptional effects of activin A in αT3-1 cells. Further analysis localized the activin-responsive region of the rat follistatin gene to the 3′-end of the first intron, between nucleotides +1784 and +1844 relative to the transcription start site.

Interestingly and in contrast to the results of the current study, it has been reported that regulatory elements located between −2165 and +2 are sufficient for Smad-dependent transcription of the human follistatin gene in HepG2 cells (40, 41). These apparently conflicting results could arise from differences in the human and rat follistatin genes or differences in the mechanisms of activation in the two cell types. The first of these possibilities is unlikely given the high degree of conservation of the follistatin gene in vertebrates at the nucleotide level. Alignment of up to 3 kb of the upstream promoter shows this region to be 95 or 79% identical between rat and mouse or human, respectively. Within this upstream region, putative Smad3 (at −1604) and Smad4 (at −895) sites identified in the rat follistatin gene are conserved in mouse and human. One or both of these could have mediated the effect of activin in HepG2 cells but neither was functional in αT3-1 cells. Remarkably, intron 1 is also highly conserved at the level of 93 or 77% sequence identity between rat and mouse or human, respectively. Moreover, the SBE1 site of intron 1 is completely conserved in all three. These observations provide a compelling argument for cell-specific mechanisms or cofactors that dictate the differential utilization of upstream or downstream Smad-binding elements and ultimately control the local concentrations of follistatin within a given microenvironment. Whether intron 1 of the rat follistatin gene is dispensable for the activation of this gene in cell types other than gonadotropes and whether the first intron of the human follistatin gene contributes to inducible expression in HepG2 or other cell types will have to be a subject of future experiments. Initial experiments, however, suggest that SBE1 of intron 1 does not significantly contribute to transcriptional activation in HepG2 and HEK293 cells treated with activin A or TGF-β whereas an intact SBE1 is necessary for the induction of both rat and mouse follistatin in gonadotrope-derived αT3-1 as well as the more differentiated LβT2 cell line (data not shown).

Activin-mediated Smad2/3 signaling has a critical role in pituitary gonadotropes to promote differential expression of FSH over LH at appropriate stages of the reproductive cycle and maintain sensitivity to GnRH by inducing FSHβ (61–63) and regulating GnRH-R expression (64, 65). Coordinate and in conjunction with feedback control mechanisms mediated by gonadal inhibin and steroids, activin induces the local production of pituitary follistatin to modulate further activin signaling and promote timely fluctuations of gonadotropins necessary for normal cycling (6, 8). In rodents, pituitary follistatin levels peak in late proestrus but are low in the morning of estrus when the activin-dependent secondary FSH surge occurs (66). Studies of the FSHβ and GnRH-R genes have highlighted the complexity of this system and begun to unravel the mechanism through which activin, GnRH, and gonadal steroids promote the hierarchical recruitment of cofactors and coordinately regulate their expression in gonadotropes. Whether some of the same factors that have been shown to cooperate with Smad2/3 at the GnRH-R promoter, such as Lhx3, Pitx-1, or AP-1 (64, 67, 68) or at the FSHβ promoter, such as Pitx1, Pitx2, Lhx3, or TALE homeodomain protein, Pbx1 and Prep1, (63, 69, 70), also exert control over follistatin expression in gonadotropes remains an open question for future studies.

Altogether, the studies described here demonstrate that a Smad-binding element in intron 1 of the rat follistatin gene mediates the transcriptional effects of activin in mouse gonadotrope-derived αT3-1 cells. The results suggest that this Smad-binding element, designated SBE1, preferentially recruits Smad3 to facilitate cell-specific expression of follistatin in response to activin A. By revealing the potential complexity of the mechanisms that may be involved in Smad-dependent regulation of the follistatin gene in different cell types, the present findings provide a framework for future studies for assessing if the mechanism defined in the current work is unique to gonadotropes. Preliminary experiments suggest that SBE1-mediated activation of the follistatin gene is dependent on the cooperation of Smad3 with cell-type restricted factors present in αT3-1 but not in HepG2 or HEK293T’ cells. Ultimately, the identification of these cell-specific factors and a better understanding of the underlying mechanisms could potentially identify tools for cell-specific manipulation of the levels of this important modulator of activin signaling.

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