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Activin Regulates βA-Subunit and Activin Receptor Messenger Ribonucleic Acid and Cellular Proliferation in Activin-Responsive Testicular Tumor Cells*

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ABSTRACT

Activin, a member of the transforming growth factor-β superfamily of growth and differentiation factors, has a number of actions in embryonic as well as adult tissues. These actions are mediated via a family of receptors containing two subtypes and at least two members of each subtype. Recent evidence demonstrates that activin-responsive cell lines containing different subsets of these receptors are valuable models for dissecting functional relationships among receptor subtype, signal transduced, and response obtained.

TT cells, derived from a $p53^{-/-}/\alpha$-$inhibin^{-/-}$ mouse testicular tumor, respond to activin by proliferating, a response that can be inhibited by follistatin (FS) treatment. Using semiquantitative RT-PCR methods, we characterized steady state messenger RNA (mRNA) levels for the inhibin/activin subunits, FS, and activin receptor subtypes under basal conditions and in the presence of activin or FS. These cells produced ample immunoreactive activin A and FS, necessitating higher treatment doses to observe any modulation of cellular proliferation. Furthermore, in the presence of exogenous activin, mRNA levels for activin receptor type IIA (ACTRIIA) and βA were significantly and profoundly suppressed. In addition, both ACTR1B and ACTRIIB were detectable and down-regulated by exogenous activin, although not to the degree observed for ACTRIIA and βA. Finally, activin treatment at the higher doses, which decreased activin receptor mRNA levels, resulted in inhibition of cellular proliferation. Taken together with previous observations, our results support the model that these tumor cells respond to an autocrine activin signal by proliferating, whereas exogenous or excess activin results in down-regulation of activin receptor and activin biosynthesis, suggesting a potential autocrine/paracrine mechanism by which activin can modulate its own signal. (*Endocrinology* **139:** 1147–1155, 1998)

THE TRANSFORMING growth factor-β (TGFβ) superfamily of growth and differentiation factors is a group of structurally related, growth regulatory proteins that appear to function in diverse biological processes, ranging from mesoderm induction and bone formation to pituitary hormone biosynthesis and reproductive physiology. Activins are homo (βA-βA or βB-βB) or heterodimeric (βA-βB) members of this superfamily, initially isolated from ovarian follicular fluid by virtue of their ability to stimulate the synthesis and release of FSH from pituitary cells in culture. Inhibins (A and B), on the other hand, are heterodimeric proteins composed of the same β-subunits found in activin complexed to a more distantly related α-subunit (1–3).

In addition to endocrine regulation of pituitary FSH release, activins and inhibins have been implicated in the local autocrine/paracrine regulation of gonadal function (4, 5). In the testis, inhibins are mainly secreted by Sertoli cells (6–9), where they appear to decrease spermatogonial mitotic activity (10) and enhance LH-stimulated testosterone production (11). Activins appear to be produced by Sertoli cells, germ cells, and immature Leydig cells (4, 6, 12, 13) and have been implicated in regulation of germ cell mitotic and differentiation activities (14, 15). Furthermore, biosynthesis of inhibin and activin subunits appears to be tightly regulated during embryogenesis as well as during defined stages of the spermatogenic cycle (13, 16–18).

Follistatin (FS), the activin-regulating protein, and the activin receptors are also expressed in a cell type- and developmental stage-specific manner (19, 20), providing additional evidence for an important regulatory role for this protein family. FS is a monomeric glycoprotein found in tissues (21, 22), human follicular fluid (23), and serum (24–26) whose only known actions are mediated through nearly irreversible binding and neutralization of activin (21, 27–28).

Members of the TGFβ superfamily act at the cellular level through a complex of two related serine/threonine kinase receptor subfamilies collectively known as type I and type II receptors (reviewed in Ref. 29). Current evidence supports a model in which ligand binds to one or more type II receptors that then phosphorylate type I receptors, which, in turn, transduce an intracellular signal (30). At least two type I and two type II activin receptors (ACTRI and ACTRII, respectively) have been biochemically and functionally characterized (29). Atlhough type I-II receptor complexes may exist in the unliganded state, complex formation is necessary for

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signal transduction (29), so that the relative numbers of each type of activin receptor on the cell surface at any moment may be a critical factor in determining the cell's responsiveness to a particular activin dose. Thus, delineating the mechanisms by which biosynthesis of these receptors is regulated could be critical for understanding the functions of activin *in vivo*.

We, therefore, undertook studies to determine whether activin regulates steady state levels of its receptor messenger RNA (mRNA) in an activin-responsive cell line derived from a testicular tumor generated in an α-inhibin/p^{53} knockout mouse (31, 32). This TT cell line was previously demonstrated to increase proliferation in response to activin, an effect that could be partially abrogated by administration of FS, suggesting that activin was critical to the maintenance of growth in these tumor cells (32). In addition to further characterizing activin and FS biosynthesis in these cells, our studies identified all four activin receptor subtypes. We further observed that activin administration acutely decreased both βA-subunit and activin receptor steady state mRNA levels while inhibiting proliferation of these cells at higher doses. Taken together, these results suggest a mechanism through which activin acts to regulate growth, an effect that can be modulated by changes in activin biosynthesis, receptor availability, and/or production and action of FS.

Materials and Methods

Cell line and growth conditions

TT cells are a clonal, testicular tumor cell line derived from a mouse deficient in both α-inhibin subunit (31) and p53 (32). Production and initial characterization demonstrated that these cells express ACTRI and ACTRII and proliferate in response to exogenous activin, whereas both basal and activin-stimulated proliferation can be inhibited by exogenous FS 288 (32). These cells were provided by Dr. Aaron Hsueh (Stanford University, Stanford, CA). To avoid potentially confounding effects of growth factors, such as insulin, that were used in previous studies (32), cells were grown in DMEM-Ham's F-12 (1:1) supplemented with 10% heat-inactivated FBS (Life Technologies, Grand Island, NY), 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulfate) at 37 C in a 5% carbon dioxide atmosphere. Except as detailed in experimental protocols, medium was replaced every 3–5 days.

Characterization of FS and activin production by TT cells

TT cells were plated into 100-mm dishes and incubated until confluent (~48 h). Medium was removed and analyzed for activin A and FS content.

Total activin A was measured using an anti-βA-subunit monoclonal antibody (E4, Serotec, Oxford, UK) (33) and procedures described by Knight *et al.* (34). The antibody was oxidized and plated as previously described (35) at 250 ng/well for use as capture antibody and biotinylated using biotin-LC (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions for detection of captured activin molecules. Antibody-coated wells were blocked with 1% BSA in 0.1 M Tris (pH 7.5) plus 0.1% sodium azide for 1 h, followed by two washes with wash buffer (0.05 M Tris plus 0.15 M NaCl, 0.1% sodium azide, and 0.05% Tween-20, pH 7.5). Samples were incubated for 10 min at 95 C with an equal volume of 20% SDS, followed by cooling and treatment with hydrogen peroxide as previously described (35). After washing the plates, 25 μl enzyme immunoassay diluent (0.1 M Tris, 10% BSA, 5% Triton X-100, and 0.1% sodium azide), 100 μl of samples or standards, and 25 μl biotinylated E4 antibody (35 ng/ml) were added. After overnight incubation at 4 C, the plates were washed, and strepavidin-AP (Boehringer Mannheim, Indianapolis, IN) was added (1:2000 dilution) for 2 h. After 10 additional washes, 100 μl *p*-nitrophenylphosphate in 0.05 M ethanolamine buffer (pH 9.8) were added and incubated at room temperature until color developed in control wells, at which time the assay was read in a microtiter plate reader (Molecular Devices, Menlo Park, CA). Standards were prepared from recombinant human activin A provided by the Genentech reagent distribution program (San Francisco, CA) and analyzed by four-parameter logistics software (SoftMax, Molecular Devices). The minimum detectable dose of activin in this assay was 5 ng/ml, determined as the dose of activin giving an absorbance reading 2 SD above the zero dose in five consecutive assays. Recovery of exogenous recombinant human activin A from culture medium was estimated at 75% over a range of activin doses with an intraassay coefficient of variation of 7%. The activin levels reported are a mean of three separate determinations.

FS was analyzed by two methods. First, total FS in conditioned medium was assessed by RIA as previously described in detail (25), using a mouse polyclonal antibody that recognizes both free and complexed FS from all species tested. Second, free FS in TT cell-conditioned medium was assessed using gel filtration chromatography as previously described (23, 24). Conditioned or unconditioned TT cell medium (100 μl) was incubated with radiolabeled activin (~200,000 cpm; SA, 35 μCi/μg) (24) for 2 h at room temperature, followed by overnight incubation at 4 C. Samples were chromatographed on a Superose (Pharmacia, Piscataway, NJ) gel filtration column (two tandem 10 × 30-cm columns). The column was calibrated with low molecular mass standards (Pharmacia), including ferritin (440,000 kDa), aldolase (158,000 kDa), BSA (66,000 kDa), and radiolabeled activin, as shown in Fig. 1. The resulting

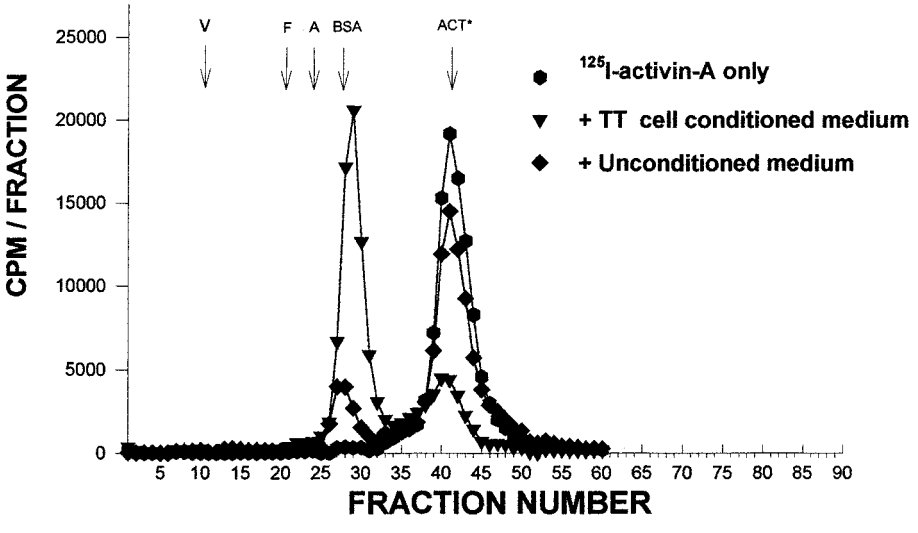

FIG. 1. Characterization of free FS in TT cell-conditioned medium by gel filtration. Radiolabeled activin eluted from a tandem Superose gel filtration column in a single peak centered at fraction 41. Preincubation of radiolabeled activin with TT cell-conditioned medium shifted the free activin peak to fraction 28 (60 kDa), the size previously observed for FS in serum (24). In contrast, unconditioned (control) medium had a small peak at the same position due to activin-binding activity in the 10% FCS that is not seen in serum-free medium (24). Therefore, TT cells secrete some free FS that is capable of binding radiolabeled activin. The molecular weight markers used to calibrate the column include ferritin (440,000 kDa; F), aldolase (158,000 kDa; A), BSA (66,000), and radiolabeled activin (ACT*). V denotes the void volume (>800,000 kDa).

activin binding (FS) peak eluted at a position similar to that previously observed in both human serum and recombinant huamn FS 315-conditioned medium (24).

Dose-response and time-course study of the effects of activin A and FS on mRNA levels

Cells were passaged at dilutions not greater than 1:5 after incubation for 3–5 min at 37 C in 0.05% trypsin and were maintained in culture in 100-mm dishes for 48 h before testing the mRNA steady state levels with or without activin or FS treatment. For time-course experiments, cells were stimulated on day 3 of culture with activin A (12.5 nM) or FS (9.5 nM) for 3, 6, 12, 24, or 48 h. These relatively high doses were selected based on dose-response studies (see below) and active doses used in earlier reports (~10 nM activin and 28 nM FS) (32), keeping in mind that these cells secrete significant amounts of both activin and FS. To examine the concentration dependence of the effect of activin on βA-subunit, βB-subunit, FS, and activin receptor mRNA levels, cells were treated on day 2 of culture with 0, 0.125, 1.25, or 12.5 nM activin A for 24 h. For both sets of experiments, cellular RNA was extracted for RT-PCR analyses.

RNA extraction and RT-PCR procedures

Total RNA was extracted using the guanidine isothiocyanate/mercaptoethanol method (36). RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining as well as monitoring absorbance at 260/280. RNA concentrations were determined from the A260 absorbance before each reverse transcription experiment. Complementary DNA (cDNA) was obtained by RT at 42 C for 30 min in a 20-μl reaction mixture containing 1 μg RNA, 0.5 mM of each deoxy (d)-NTP, 5 μM oligo(deoxythymidine), 25 U SuperScript (Life Technologies), and 14 U ribonuclease inhibitor (Promega). The PCR reaction was performed in a volume of 50 μl containing 1 × PCR buffer A (Fisher *Taq* buffer, Fisher Scientific, Fairlawn, NJ), 5 mM $MgCl_2$, 0.2 mM of each dNTP, 3 μM each of 5′- and 3′-primers, 2.5 U *Taq* polymerase (Fisher), and 2 μCi nucleotide triphosphate ([^{32}P]dCTP) to which 10 μl of the RT reaction product were added. Primer sequence and location as well as expected product size are listed in Table 1. The amplification profile involved preincubation at 95 C for 3 min, denaturation at 95 C for 0.5 min, primer annealing at 50 C for 1.5 min, and extension at 72 C for 1.5 min. In initial experiments, cycle number was optimized for each target so that signals were always in the exponential portion of the amplification curve (35 cycles for all, except 24 cycles for β-actin, and 40 cycles for FS). Ten microliters of the PCR reaction were electrophoresed in 5% polyacrylamide gels in Tris-borate-EDTA buffer. Autoradiography was carried out for 12 h at room temperature, and the relative concentration of each mRNA was determined by densitometric scanning of the autoradiograms and normalization to the β-actin signal for each sample. As we observed no difference in the loading on replicate gels in preliminary experiments, each RT-PCR was run on a single gel. PCR products were verified by Southern blot using an internal oligonucleotide as a probe (see Table 1).

For ACTR1B and ACTRIIB targets, which were initially undetectable under the above conditions, a more sensitive strategy was devised. RNA was extracted using Trizol (Life Technologies), and 1 μg RNA was first treated with deoxyribonuclease, then with superscript RT, followed by ribonuclease H according to the manufacturer's (Life Technologies) instructions. Briefly, the RT conditions were similar to those described above, except that ribonuclease inhibitor was not used. The PCR reactions were performed in 25 μl 1 × PCR buffer A, 0.2 mM of each dNTP, 0.5 μM of each specific primer (see Table 1), 0.25 μl *Taq*, and 0.1 μCi [^{32}P]dCTP. Under these conditions, PCR reactions were in the exponential range for: 1) actin, 1:10 RT dilution and 21 cycles; 2) ACTRIIB, 1:10 RT dilution and 31 cycles; and 3) ACTR1B, 1:2 RT dilution and 31 cycles. The identities of the resulting PCR products were verified for both by Southern blot as described below.

Southern blot analyses

PCR product identity was verified by Southern blot using synthetic oligos located internal to the two PCR primers (see Table 1). PCR-amplified products without radionuclide were fractionated on a 1.5% agarose gel and visualized by UV transillumination, and the product sizes were estimated by comparison to mol wt markers (PGEM, Promega). The gels were then denatured in 50 mM NaOH-1.5 M NaCl for 30 min and neutralized in 1 M Tris-1.5 M NaCl. The DNA was transferred by capillary action to a nylon membrane (Micron Separations, Westborough, MA) in 20 × SSC (3.0 M NaCl and 0.3 M sodium citrate, pH 7.0) and subjected to Southern blot analyses using [^{32}P]dATP-labeled oligonucleotide probes. Hybridization was performed with each labeled oligonucleotide in 5–10 ml of a solution containing 5 × SSC, 1 × Denhart's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA overnight at 42 C. The membranes were washed twice in 0.2 × SSC at 67 C for 15 min each time, followed by autoradiography.

TABLE 1. Sequence, location, and expected fragment size for synthetic oligos used for PCR and Southern blotting

Target	Primer	Sequence	Position	Size	Ref. no.
Inhibin-βA	5′	ctgaa cgcga tcaga aagct	486–506	1014	49
	3′	tcctc cacga tcatg ttctg	1482–1501		
	Southern	cgggg ccata gcccc tttgc caacc tcaaa tcg	1370–1403		
Inhibin-βB	5′	ccagc ctctg ttgca ggcaa	44–63	301	50
	3′	gatca tgttg ggcac atccc	326–345		
	Southern	tttcg gctca tcggc tggaa cgact ggatc at	79–110		
Follistatin	5′	aaaga aacgt gtgag aacgt tgga	274–298	396	51
	3′	caatg gatct gccca gcaag	675–694		
	Southern	gggat gtttt ttgtc caggc a	506–526		
ACTRIIA	5′	gaaaa tggga gctgc tgcaa agttg	170–194	508	52
	3′	gtagg ccatc ttgtg atgcc tgtac	653–677		
	Southern	aaaca aggtt tgttg gctgg atgat atcaa ctg	396–429		
ACRRIIB	5′	aactg ggagc tggag cgcac caac	145–169	222	47
	3′	gaagt tgcct tcgca gcagc agaa	343–367		
	Southern	aagaa gggct gctgg ctaga tgact tcaat	263–292		
ACTRIA	5′	gccca aggtc aaccc caaac tct	178–200	265	53
	3′	ggatt ttcct ttagt gggca gct	420–442		
	Southern	ggcca gcagt gcttt tcctc actga gcatc aat	251–283		
ACTRIB	5′	accag ctgcc tccag gccaa cta	109–131	245	53
	3′	gtgct caggc tcctt gaggt gac	322–354		
	Southern	tgcct gagct cggcg gacct gcgcc aacac cca	250–282		
β-Actin	5′	atcct gcgtc tggac ctg	564–581	551	54
	3′	gctga tccac atctg ctg	1098–1115		

TT cell proliferation studies

TT cells were plated at 30,000–50,000/well in six-well culture dishes in 1–2 ml medium containing either 5% or 10% FCS (no difference was observed with respect to activin response). Increasing volumes of activin containing medium (see below) or an equivalent amount of control medium (wild type 293 cell-conditioned medium) were added to triplicate wells. The cells were further incubated for 24, 48, 72, or 96 h, at which time they were removed (3–5 min of trypsinization) from the plates and counted in a hemocytometer using trypan blue to distinguish live cells. After correcting for dilution, live cell number was plotted *vs.* time in culture for each dose of activin or control medium. Results were initially calculated as the number of live, activin-treated cells/number of live, control medium-treated cells × 100.

Recombinant activin-containing conditioned medium

Preliminary experiments with lower doses (1–100 ng/ml) of purified activin from Genentech did not have a significant effect on TT cell proliferation, in contrast to previous observations of TT cells (32). To examine higher doses of activin with potentially more bioactivity, we used conditioned medium from 293 cells stably transfected with a human inhibin/activin βA-subunit cDNA (provided by Genentech). Conditioned medium was concentrated 20-fold using a stirred cell apparatus with a 10-kDa cut-off membrane (Filtron, Northborough, MA) and aliquoted. Aliquots were calibrated against purified recombinant activin from Genentech (lot 18395) and NIH (lot 15365–36) using a previously described, solid phase, ligand binding assay (SILBA) (28) as well as by RIA. The RIA used purified activin from Genentech as radioligand and a rabbit antibody raised to recombinant inhibin A (M863-8) diluted in PBS to 1:500. The iodination protocol and remaining assay steps were previously reported for inhibin (23, 37). Using both RIA and SILBA, the 20-fold concentrated medium, designated MGH-ACT-2, contains 30 μg/ml activin immunoactivity and FS binding activity with approximately the same bioactivity as the activin distributed by the NIH (data not shown). As a control, medium conditioned by wild-type 293 cells was concentrated 20-fold and added to the cultures at volumes equal to those of the activin-containing medium.

Data analysis

Densitometric results (RT-PCR experiments) were normalized to the β-actin level for each sample and are reported as the mean ± SE for two or three independent experiments. For the dose-response studies (Fig. 4), the results for each dose of activin were compared with those for the zero dose sample. For time-course studies (Figs. 3, 5, and 6), the effects of activin or FS at each time point were compared with an untreated control sample incubated for the same duration. All statistical differences for these experiments were determined using *t* test, comparing each treatment to the no treatment control. Finally, Fig. 7 represents data collected from eight different experiments, each covering an overlapping, but not complete, set of doses such that each dose represents observations ranging from 1–5. Experiments were combined by expressing results for each experiment as a percentage of the control value, and the composite for eight experiments is shown in Fig. 7. Significant differences were determined using Student's *t* test as indicated by letter codes, with $P < 0.05$ considered significant for all experiments.

Results

Secretion of FS and activin A by TT cells

When analyzed by specific RIA for total FS, TT cell-conditioned medium contained 10.1 ng/ml (0.28 nM) FS, whereas unconditioned medium contained 5.7 ng/ml, for a net FS of 4.1 ng/ml. To determine whether this FS was functional as an activin-binding protein (BP), TT cell-conditioned medium (100 μl) was incubated with radiolabeled activin and chromatographed under conditions that allow separation of FS-activin complex from free activin (24). TT cell-conditioned medium contained a large FS peak at fraction 29 (Fig. 1), consistent with the activin BP previously observed in human serum at approximately 60 kDa (24). In contrast, the BP in unconditioned TT medium (control) was much smaller and was probably derived from the FCS (see RIA results above). Thus, it would appear that at least some free FS is secreted by TT cells.

Using a specific two-site activin A immunoassay that measures total activin (free activin plus activin bound to FS), TT cells secrete approximately 15 ng/ml (0.5 nM) activin A/48 h or 4.6 ± 3.4 ng/ml/1.3 million cells·24 h (n = 3). No activin immunoactivity was detected in unconditioned medium.

Steady state mRNA levels

TT cell expression of inhibin/activin βA- and βB-subunits, FS, and activin receptor mRNA by TT cells was examined using RT-PCR analysis. The identity of the amplified PCR products was demonstrated by Southern blotting. All PCR products were analyzed on a 1.2% agarose gel and electrophoresed adjacent to a lane with DNA markers (Fig. 2A). After transfer and hybridization, each of these PCR products was specifically recognized by an internal 33-mer synthetic oligo probe that did not bind to the adjacent marker DNA (Fig. 2B).

In the absence of activin treatment, no modification was found in βA- or βB-subunits, FS, ACTRIB, ACTRIIB, or ACTRIIA mRNA levels at 3, 6, 12, 24, and 48 h of incubation. However, a significant 3-fold increase in ACTR1A steady state mRNA levels was observed starting at 3 h of incubation (Fig. 3).

Regulation of inhibin/activin β-subunit and activin receptor mRNA levels by activin A

Activin's effects on ACTRIIA and βA mRNA were significant only for the highest dose (12.5 nM), achieving suppression to 75% and 63% of the untreated control values, respectively (Fig. 4A). This dose of activin was even more effective in reducing ACTRIIB and ACTRIB mRNA levels, achieving suppression to 30% and 20% of control, respectively (Fig. 4B).

The time course for the effects of 12.5 nM activin A treatment on the expression of inhibin/activin subunit and activin receptor mRNA was analyzed next. Treatment with activin resulted in 50% and 90% suppression of ACTRIIA steady state mRNA levels at 24 ($P < 0.01$) h of incubation (Fig. 5A). Similarly, this treatment caused a 75% reduction ($P < 0.02$) of βA-subunit mRNA at 24 h (Fig. 5A). For both targets, mRNA levels remained below control levels at 48 h of incubation, although this difference was not significant.

This 12.5-nM dose of activin A resulted in a maximal 40% suppression of ACTRIIB mRNA (Fig. 5B) at both 24 and 48 h ($P < 0.05$ and $P < 0.001$, respectively) as well as 50% and 30% suppressions of ACTR1B mRNA at 24 and 48 h ($P < 0.01$ and $P < 0.05$, respectively). Thus, ACTR1A was the only activin receptor subtype whose expression did not appear to be modulated by treatment with activin A.

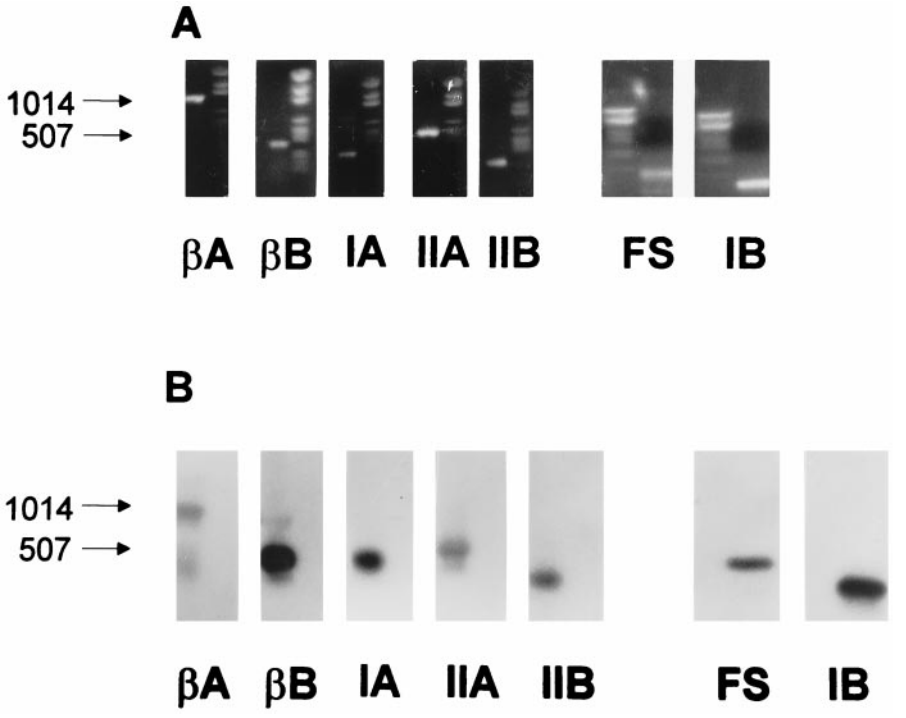

FIG. 2. Verification of PCR fragment identity by Southern blot. A, Ethidium bromide-stained agarose gel depicting each PCR product and a corresponding marker lane. The approximate sizes, as estimated from markers, are indicated on the *left*. B, Southern blot of the gel shown in A after transfer to Nytran. ^{32}P-Labeled oligonucleotide probes (as indicated in Table 1) were hybridized overnight, washed at high stringency, and autoradiographed from 1–24 h. Specific recognition of the PCR product by Southern probe without detection of adjacent marker DNA demonstrates the specificity of the probes and verifies all PCR targets as authentic. The approximate sizes of the bands are indicated on the *left*.

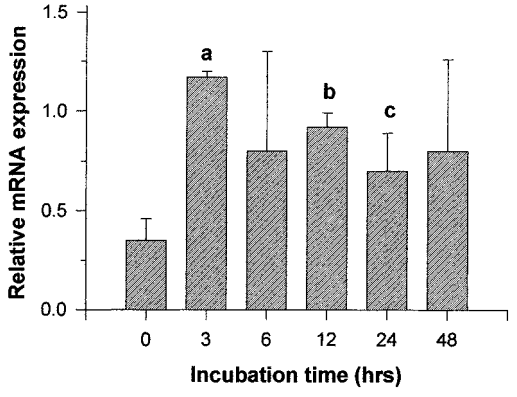

FIG. 3. Time course for basal expression of ACTRIA. Steady state mRNA for ACTRIA was examined under basal conditions for up to 48 h. ACTRIA mRNA levels increased significantly by 3 h and remained elevated, although the 6 and 48 h points were not statistically significant. No other target showed any significant change under basal conditions. Results are the mean ± SE of three separate experiments, expressed relative to β-actin for each time point. Significant differences from the zero time sample are indicated (a, $P < 0.002$; b, $P < 0.01$; c, $P < 0.05$).

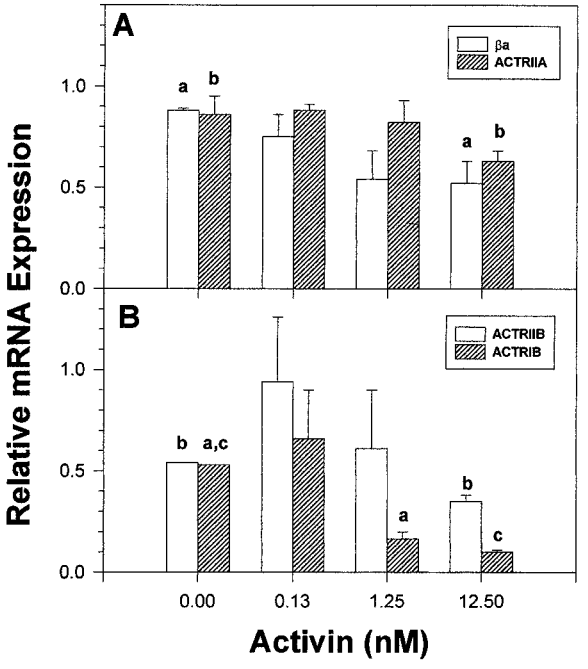

FIG. 4. Effects of activin dose on activin subunit and receptor mRNA levels in TT cells. A, TT cells were treated for 24 h with increasing doses of activin A. Only the 12.5 nM dose resulted in significant changes, with βA-subunit suppressed to 63% of the untreated control value ($P < 0.02$; a), whereas ACTRIIA was reduced to approximately 75% of the untreated control value ($P < 0.05$; b). B, After 24 h of incubation, expression of ACTRIIB mRNA was suppressed 30% ($P < 0.05$; b) with 12.5 nM activin A, whereas ACTR1B mRNA was decreased 65% ($P < 0.05$; a) at 1.25 nM and 80% ($P < 0.01$; c) at 12.5 nM activin A compared with the untreated control value. For both graphs, results are expressed relative to β-actin and are plotted as the mean ± SE for three separate experiments.

Regulation of β-subunit, activin receptors, and FS mRNA by recombinant human FS 288 treatment

Treatment of TT cells with 9 nM recombinant human FS 288 for 24 h induced a 1.5-fold ($P < 0.05$) increase in the expression of both ACTRIIA and FS mRNA (Fig. 6). Interestingly, a 25% reduction in βB-subunit mRNA (12 h after activin treatment) was found, although this effect did not reach statistical significance (data not shown). FS treatment produced a slight, but consistent, increase in the expression of ACTR1B and ACTRIIB mRNA, but this was only signif-

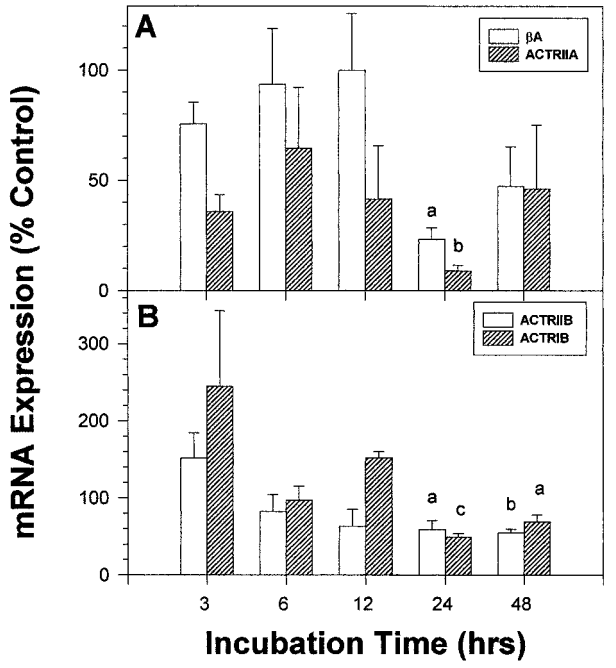

FIG. 5. Effects of incubation time in the presence of activin A on activin subunit and receptor mRNA expression. A, Treatment with 12.5 nM activin caused significant reductions in ACTRIIA (90%; $P < 0.01$; a) and βA-subunit (75%; $P < 0.02$; b) mRNA levels at 24 h. Results are expressed relative to β-actin at each time point and are plotted as the mean ± SE for three independent experiments, expressed as a percentage of untreated control value at each time point. Significance was determined by *t* test *vs.* the zero time control. B, At the 12.5 nM activin A dose, steady state levels of both ACTRIB and ACTRIIB mRNA were significantly suppressed at 24–48 h (a, $P < 0.05$; b, $P < 0.001$; c, $P < 0.01$). Each time or treatment point represents the mean ± SE of three independent experiments after normalization to β-actin, expressed relative to the untreated control value for each time point. Significant differences from the zero time point were determined.

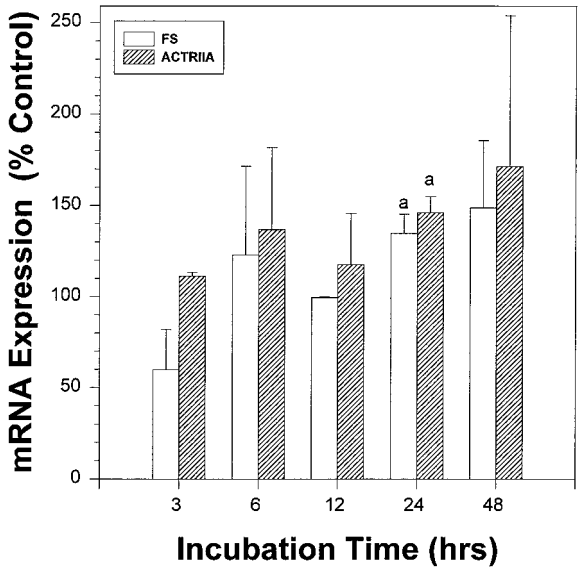

FIG. 6. Effect of incubation in the presence of FS on ACTRIIA and FS mRNA expression in TT cells. Treatment of TT cells with 9 nM FS 288 resulted in a maximal 1.5-fold increase in both FS and ACTRIIA mRNA steady state levels ($P < 0.05$; a) at 24 h compared with that in the untreated control. No FS effects were observed for any other PCR target. Results are the mean ± SE of three independent experiments, normalized to β-actin and expressed as a percentage of the untreated control value. Significance was determined by *t* test from the zero time control.

icant at 48 h (data not shown). No significant effects of FS treatment were observed for activin βA and ACTR1A mRNA.

TT cell proliferation studies

At activin doses of 30 nM or greater, significant inhibition of cellular proliferation was observed after 72 and 96 h of treatment (Fig. 7). These doses exceed those at which we observed significant decreases in activin subunit and its receptor mRNA levels. In contrast, we were unable to document a statistically significant increase in proliferation at any activin dose or incubation time under the culture conditions used in these studies. This inhibitory action of activin was not due to toxicity from the conditioned medium, as equivalent amounts of wild-type 293 cell-conditioned medium had no effect, and only live cells were counted. Thus, at higher activin doses, especially those at which ACTRIIA and βA-subunit were also reduced, we observed a significant and dramatic inhibition of cellular proliferation.

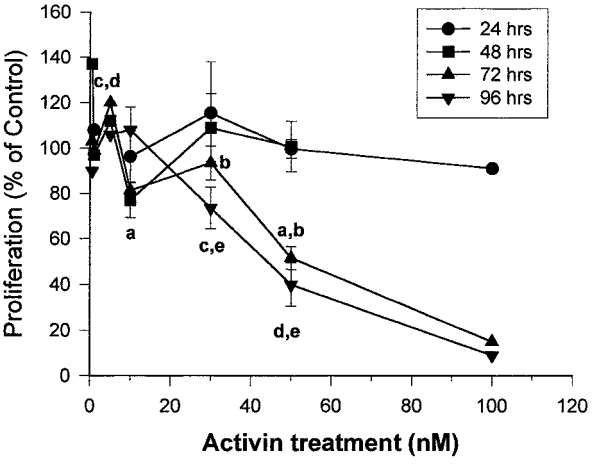

FIG. 7. Effect of activin A treatment on TT cell proliferation. The number of viable TT cells was determined by cell counting every 24 h for 4 days in the absence or presence of activin A, ranging from 0–100 nM. Lower doses of activin had little effect, but higher doses significantly inhibited cellular proliferation, an effect that was maximal at 72–96 h. The number of observations at each dose and time point range from 1 (0.05 nM activin) to 5 (10 and 50 nM doses) and were pooled from eight separate experiments covering different subsets of activin doses. Results are calculated as the mean ± SE for each dose at each time point, and are expressed as a percentage of the values in control plates treated with identical amounts of concentrated 293 cell medium conditioned by wild-type 293 cells to control for possible nonactivin-mediated effects (see *Materials and Methods*). Significant differences include: $P < 0.005$ (a) and $P < 0.01$ (b) for 72 h of incubation; and $P < 0.05$ (c), $P < 0.001$ (d), and $P < 0.05$ (e) for 96 h of incubation.

Discussion

Activin can influence cellular proliferation, differentiation, or death in a number of tissues and cell types, yet the mech-

anism(s) by which these actions are manifested is only now being elucidated (29). Thus, activin-responsive cell lines provide an invaluable tool for dissecting the series of events that is induced upon activin signaling, including receptor binding, oligomerization, phosphorylation, and activation of downstream pathways involved with regulation of proliferation, differentiation, and apoptosis (38–40). The TT testicular tumor cell line, cloned from a mouse in which α-inhibin and p53 were deleted by homologous recombination (31), represents one such tool.

Our RT-PCR results demonstrate that these cells express mRNA for all four activin receptor subtypes (29) characterized to date, including ACTRIIA, ACTRIIB, ACTRIA, and ACTRIB. In addition, we quantitated the amounts of activin and FS secreted by these cells and determined the approximate amount of free FS in the medium. Using semiquantitative RT-PCR analysis, we also observed that exogenous activin regulates its own and its receptor mRNA levels. These observations extend the earlier studies by Shikone *et al.* (32), who demonstrated that TT cells contain βA, βB, and FS mRNA as well as βA-subunit and type I and II activin receptor proteins.

When treated with 12.5 nM activin, a dose necessary to overcome endogenous activin and FS production, TT cell expression of ACTRIIA mRNA was decreased by 90%, whereas βA-subunit mRNA was decreased by 75%. Similarly, this dose of activin had a significant negative influence on ACTR1B and ACTRIIB mRNA levels, decreasing steady state levels by 50% at 24 h. Although we did not observe consistent effects of activin on TT cell proliferation at this 12.5 nM activin dose, larger doses significantly inhibited cellular proliferation in a dose-dependent fashion, suggesting that this decrease in mRNA steady state levels in response to activin was functional at the cellular effector level. Furthermore, our results are consistent with the significant 65% reduction in testicular ACTRIIA mRNA observed *in vivo* in α-inhibin-deficient mice with elevated circulating activin levels and βA-subunit production (41, 42). If reductions in activin receptor and βA-subunit mRNAs are reflected in secreted protein, our results suggest that the activin signaling system might become down-regulated in response to high levels of exogenous or endogenous activin and that this down-regulation is manifested by inhibition or removal of growth factor support for cellular proliferation. In addition, this decrease in activin receptor or βA-subunit mRNA levels may have important inhibitory consequences on testicular steroidogenesis and spermatogenesis despite elevated activin levels. Indeed, Leydig cell numbers decrease and spermatogenic regression occurs in parallel with tumor growth in α-inhibin-deficient mice with increased circulating activin levels (41).

Treatment with the activin BP FS 288 reversed activin's effect on both basal (endogenous) and activin-stimulated proliferation (32). Consistent with this, we observed that FS treatment caused slight increases in both activin receptor and FS mRNA levels. We also observed a significant increase in ACTR1A mRNA levels in untreated cells between 3 and 24 h of incubation. This latter finding suggests that endogenous factors can regulate ACTR1A mRNA expression. Although this endogenous factor could be activin itself, our failure to observe changes in ACTR1A mRNA levels with high doses of activin or FS indicate that other growth regulatory proteins might be responsible.

Using a specific, two-site, total (free plus FS bound) activin A immunoassay, we determined that these cells produce significant amounts of activin on the order of 15 ng/ml conditioned medium/48 h. Immunoreactive total FS (free FS plus FS bound to activin) in a separate batch of conditioned medium was 4.4 ng/ml, suggesting that free activin, *i.e.* that secreted in excess of FS, might be secreted by TT cells. This would be consistent with the observation by Shikone *et al.* (32) that addition of FS could suppress basal as well as activin-stimulated proliferation. On the other hand, the peak of radiolabeled activin complexed with FS, as determined by chromatographic analysis of TT cell conditioned medium, suggests that at least some free FS is also secreted by TT cells. As this FS-activin peak eluted at a position similar to that observed in human serum and recombinant human FS 315-conditioned medium (24), the FS secreted by TT cells might contain the 315 form. Although recombinant human FS 288 was previously demonstrated to bind activin with nearly irreversible kinetics (28), FS 315 has been described to bind activin at a lower affinity (43). Thus, the detection of a FS-activin complex in TT cell-conditioned medium might result from exchange of bound unlabeled activin for radiolabeled activin during the incubation before chromatography, rather than representing only free FS. Alternatively, both the total FS RIA and the activin A enzyme-linked immunosorbent assay use human standards, so the mass estimation of secreted mouse FS and activin from TT cells may be inaccurate due to species specificity of the antibodies used in either assay. Nevertheless, our results demonstrate significant production of both activin and FS by TT cells.

Previous studies using TT cells demonstrated that due to the production and secretion of both activin and FS, the use of relatively high doses of activin was necessary to obtain statistically significant increases in cellular proliferation rates (*e.g.* 1 nM) (32). However, under the growth conditions employed in the present studies, stimulation of proliferation was not observed for any dose of activin, nor did we observe inhibition of basal proliferation for any dose of FS up to 30 nM. The effects of FS and activin on TT cell proliferation previously observed (32) may have resulted from their additional supplementation with growth factors such as insulin and epidermal growth factor. Nevertheless, at higher doses of activin, we consistently observed significant inhibition of proliferation as well as a robust and significant decrease in βA and activin receptor mRNA levels.

Taken together, the two TT cell studies suggest that activin might influence cellular proliferation in a biphasic manner. Similar biphasic effects on cell growth were observed in response to activin for porcine thyroid cells (44) as well as for connective tissue cells in response to TGFβ (45). Perhaps more importantly, this biphasic response to activin has also been reported for testicular cultures, particularly for seminiferous epithelium at stages VIIa–VIIc (46). Interestingly, this phase change in activin action occurred at doses similar to those resulting in reduced βA and activin receptor mRNA levels in TT cells. Thus, if changes in mRNA correlate with protein expression, our results suggest a mechanism by

which activin can modulate its activity level by down-regulating its own and its receptor biosynthesis, thereby suppressing the autocrine growth signal. Alternatively, high, chronic activin levels might induce apoptosis, as recently observed in a human prostate cancer cell line, LNCaP (40), resulting in the observed decrease in proliferation at the activin doses used in this study.

Northern blot analysis of normal mouse testis revealed an equal abundance of ACTRIIA and ACTRIIB (47), whereas a greater abundance of ACTRIIA was detected compared with that of ACTRIIB in testis from adult rat (48). In this study, we identified all four activin receptor subtypes and determined that at least three of them, ACTR1B, ACTRIIA, and ACTRIIB, are regulated by ligand. Differences in the activin receptor content of various activin-responsive cell lines have been recently demonstrated (38, 39). Interestingly, those studies identified ACTRIB as the dominant activin signal transducer. If the same relationship holds for TT cells, then expression of at least the major activin-signaling receptor subtypes appears to be regulated by activin. It would be of interest to determine the prevalence of each possible combination of ACTRI and ACTRII subtypes in primary tumors or in other tumor cell lines that respond to activin with proliferation to understand the potential role of activin in normal reproduction as well as in pathogenesis of infertility and carcinoma. Our results also suggest that TT cells will be an excellent model in which to dissect the intricate relationships among receptor subtype expression, production of autocrine growth factors, and inhibitory binding protein modulation of signal transduction.

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