Inhibition of Prolactin Gene Transcription by Transforming Growth Factor-β in GH3 Cells

Beverly C. Delidow, William M. Billis, Puja Agarwal, and Bruce A. White

Department of Anatomy
University of Connecticut Health Center
Farmington, Connecticut 06030

Transforming growth factor-β (TGFβ) is a member of a large family of growth factors, several of which regulate pituitary function. TGFβ has recently been reported to reduce PRL production by GH cells. We have examined the effect of TGFβ on PRL gene expression in rat pituitary tumor GH3 cells. TGFβ1 or TGFβ2 reduced both basal and Ca2+-stimulated PRL mRNA levels. This inhibition was specific, as the mRNA levels for GH, glucose-regulated protein 78, and histone-3 were unaffected by TGFβ. Inhibition of PRL gene expression by TGFβ was dose dependent in the range of 0.5–10 ng/ml. TGFβ inhibited run-on PRL gene transcription in nuclei from treated cells to the same extent that it reduced PRL mRNA levels, indicating a transcriptional mechanism of action. However, TGFβ did not affect Pit-1 mRNA levels or run-on transcription of the Pit-1 gene. Thus, TGFβ does not appear to act through modification of Pit-1 gene expression. The PRL promoter contains two regions of homology, with a consensus sequence found in the promoters of other TGFβ-inhibited genes. These findings are consistent with other studies that have demonstrated transcriptional repression by TGFβ. The potency and specificity of the effects of TGFβ on PRL gene expression suggest that it may be a physiological regulator of lactotroph function. (Molecular Endocrinology 5: 1716–1722, 1991)

INTRODUCTION

PRL is unique among the pituitary hormones in that it is primarily under inhibitory control. In the absence of tonic inhibition from the hypothalamus, PRL is produced and secreted at a high basal rate (1). PRL is also produced at a high basal rate in various pituitary tumor cell lines (e.g. GH3 and GH cell lines) (2). This high basal rate of PRL gene expression is dependent on calcium in both GH3 cells (3) and primary pituitary cultures (4). Studies have indicated that Ca2+ stimulates PRL gene expression at both transcriptional (5–7) and posttranscriptional levels (7). In addition to the Ca2+-dependent enhancement of basal PRL gene expression, the PRL gene is regulated by several hormones, neurotransmitters, and growth factors. The primary inhibitor of PRL production in vivo is dopamine (1). Somatostatin and glucocorticoids also inhibit PRL gene expression (8–10). In addition, there are a number of factors capable of stimulating PRL synthesis and release, including TRH (11), vasoactive intestinal peptide (12), and estrogen (10, 13, 14). Serum augments PRL production in cultured pituitary cells, and at least part of this stimulation is due to growth factors. Among these are fibroblast growth factor and epidermal growth factor (EGF), which are found in the pituitary (15, 16). Both EGF and fibroblast growth factor stimulate PRL gene expression (17, 18). Transforming growth factor-β (TGFβ), which is structurally related to EGF and binds to the same receptor, is also produced by lactotrophs (19) and stimulates PRL production (20).

In studying the regulation of several aspects of GH3 cell function by serum factors, we examined the effects of another growth factor, TGFβ, on PRL mRNA levels. TGFβ is a member of a large family of growth factors with wide-ranging effects, including pituitary hormone regulation (for reviews, see Refs. 21 and 22). There are three forms of TGFβ found in mammals as well as a fourth in the chicken (20). TGFβ has broad effects on cell growth and extracellular matrix deposition, and appears to be very important for the differentiation of some tissues during development (21, 22). Other members of this family are the developmental regulators Mullerian inhibiting substance (25), bone morphogenetic proteins (26), Xenopus mesoderm-inducing factor (27), and the Drosophila homeotic gene product decapentaplegic (28). A third subset of the TGFβ family includes the inhibins and activins (29), which are gonadal regulators of pituitary function. Inhibins and activins inhibit or stimulate, respectively, the production of FSH by gonadotrophs without affecting LH. Activin is also able to inhibit secretion of GH and ACTH by primary cultures of pituitary cells, an effect that is antagonized by inhibin (30).
TGFβ inhibits the ability of cultured fibroblasts to express the mRNA for mitogen-regulated protein/proliferin (31). Mitogen-regulated protein/proliferin is a member of the PRL/GH/placental lactogen gene family with considerable structural similarity to PRL (32, 33). Receptors that bind several members of the TGFβ family have been detected on GH3 cells (34). Ramsdell (20) recently reported that TGFβ inhibited proliferation of GH4 pituitary cells, and that TGFβ and activin decreased PRL production. We now report that TGFβ is a specific and potent inhibitor of both basal and Ca2+-stimulated PRL gene transcription in GH3 cells.

RESULTS

TGFβ Inhibits PRL Gene Expression

PRL mRNA declines to low levels in GH3 cells cultured in serum-free medium (SFM) for 24 h. As shown previously (3, 7), addition of 0.5 mM CaCl2 to SFM specifically increased PRL mRNA by several fold (Fig. 1A). The addition of 10 ng/ml of either TGFβ1 or TGFβ2 to GH3 cell cultures reduced basal PRL expression in SFM by 40% and almost completely inhibited the ability of Ca2+ to increase PRL mRNA levels (TGFβ1, 76% inhibition; TGFβ2, 72% inhibition). The effects of TGFβ1 and TGFβ2 were specific for PRL, as there was no change in the mRNA levels of GH (Fig. 1A). TGFβ treatment also lowered basal PRL mRNA levels by 35% in cells cultured in normal serum-supplemented growth medium (Fig. 1B). This extent of inhibition was the same as that in cells cultured SFM plus Ca2+ (Fig. 1B), even though growth cultures contained less PRL mRNA than Ca2+-treated cells.

Treatment of GH3 cells with doses of TGFβ ranging from 0.1–10 ng/ml (4–400 pM; Fig. 1C) showed that TGFβ is a potent inhibitor of PRL mRNA expression. Inhibition was first detectable at 0.5 ng/ml and is nearly complete at 10 ng/ml. There was no effect of TGFβ on the expression of GH, Pit-1 (see below), histone-3, or glucose-regulated protein 78 (GRP78) mRNAs over a dose range of 0.02–10 ng/ml (data not shown). The responsiveness of an independently derived pituitary tumor cell line, 235–1 cells, to TGFβ was also examined. The 235–1 cells display high levels of PRL production, but have been shown to be unresponsive to TRH and estrogen (35). In two separate experiments, TGFβ had no effect on PRL mRNA levels in 235–1 cells (data not shown).

To determine whether TGFβ inhibited PRL gene expression in GH3 cells at the transcriptional level, run-on transcription assays were performed using nuclei from cells that were either untreated (SFM control) or levels by TGFβ. GH3 cells were treated with 0.5 mM Ca2+ in the presence of increasing doses of TGFβ, from 0.1–10.0 ng/ml (4–400 pm). Values are expressed as the fold induction over the SFM control and are the average ± range of duplicate dots from duplicate cultures.
treated for 20 h with Ca\(^{2+}\) or Ca\(^{2+}\) plus 5 ng/ml TGF\(\beta\). Cytoplasmic RNA was isolated from the cells in each treatment group at the time of nuclear isolation, and PRL, GH, Pit-1, and GRP78 mRNA levels were analyzed by Northern blot hybridization. Two separate experiments produced similar results; the results of one experiment are shown in Fig. 2. As a control for inhibition of PRL gene transcription, cells were also treated with Ca\(^{2+}\) plus 100 nM dexamethasone (Dex) (9, 10). As expected, Dex decreased PRL gene transcription to 0.6-fold of that in the SFM control and reduced PRL mRNA levels by about 80%. Dex also induced a small increase in the levels of GH mRNA, as previously described (36).

PRL gene transcription in Ca\(^{2+}\)-treated cells was 1.3-fold of that in SFM controls (Fig. 2, A and C). In contrast to this small change in PRL gene transcription and consistent with previous findings (7), Ca\(^{2+}\) induced a 7.0-fold increase in the steady state levels of PRL mRNA (Fig. 2B and C). TGF\(\beta\) reduced Ca\(^{2+}\)-stimulated PRL gene transcription by 50%, to a level 0.7-fold of that in the serum-free control, and inhibited the Ca\(^{2+}\)-induced increase in PRL mRNA levels by 64%. Again, the effect of TGF\(\beta\) was specific for PRL, as mRNA levels for GH, Pit-1 (see below also), and GRP78 were not significantly changed by TGF\(\beta\).

Nuclear RNA isolated from GH3 cells treated with Ca\(^{2+}\) for 24 h in the presence or absence of 5 ng/ml TGF\(\beta\) was subjected to Northern blot analysis. In support of a transcriptional effect of TGF\(\beta\), there was a proportional decrease in both the mature PRL mRNA and nuclear PRL mRNA precursors in RNA from TGF\(\beta\)-treated cells (Fig. 2D).

Time-course studies indicate that a decrease in PRL mRNA levels can be observed as soon as 3–6 h (Fig. 3) after the addition of TGF\(\beta\) to culture medium. Inhibition was maximal at 12–24 h. Since widely divergent values have been reported for PRL mRNA stability (7, 37), it is difficult to state whether this relatively rapid decrease in PRL mRNA is due solely to transcriptional repression or to an additional destabilizing effect of TGF\(\beta\) on PRL mRNA. Although we have not performed

---

**Fig. 2. Effects of TGF\(\beta\) on PRL Gene Transcription and Cytoplasmic and Nuclear PRL RNA Levels**

GH3 cells were cultured in SFM plus 0.5 mM CaCl\(_2\) in the presence and absence of 5 ng/ml TGF\(\beta\) or 100 nM Dex for 20 h. Nuclei were then isolated and immediately used in run-on transcription reactions. RNA was isolated from the cytoplasmic lysates and analyzed by Northern blot hybridization. A. Transcription of the PRL gene, as measured by hybridization of *in vitro* run-on RNA to PRL cDNA dots. Data were normalized by comparing PRL hybridization to hybridization to control plasmid DNA (Bluescript; KS+). B. Northern blot hybridization analysis of cytoplasmic RNA from the same experiments. Equivalent amounts of cytoplasmic mRNA (10 \(\mu\)g) were electrophoresed through 1% agarose and transferred to nitrocellulose filters. Identical blots were probed for PRL, GH, Pit-1, and GRP78. Autoradiograms were quantified by scanning densitometry, and the values were normalized to the levels of GRP78 mRNA in each sample. C. Graphic representation of the PRL data from A and B. Normalized PRL mRNA levels are expressed relative to the SFM controls. D. Analysis of nuclear PRL RNA in GH3 cells treated with Ca\(^{2+}\) plus TGF\(\beta\). GH3 cells were cultured in SFM plus Ca\(^{2+}\) in the presence and absence of 5 ng/ml TGF\(\beta\) for 24 h. Nuclear RNA was isolated and subjected to Northern blot hybridization analysis for PRL, using a probe specific for PRL exons 1–5 (see Materials and Methods). The heavy arrow indicates the position of the mature 1.5-kilobase PRL mRNA; smaller arrows indicate the positions of nuclear precursors. The smaller panel shows a lighter exposure of the 1-kilobase bands.
TGF/β Inhibits PRL Gene Transcription

Fig. 3. Time Course of TGF/β Repression of PRL mRNA Levels
Duplicate cultures of GH3 cells were treated overnight in SFM plus 0.5 mM Ca²⁺. TGF/β (5 ng/ml) was added the following morning, and cells were collected at the indicated times for cytoplasmic dot hybridization analysis of PRL mRNA levels. The values given are the average ± range for duplicate dots from duplicate cultures and are expressed as a percentage of the 0 h SFM plus Ca²⁺ control. □, Values for cells treated with SFM plus Ca²⁺ alone (0 and 24 h); □, those for TGF/β-treated cultures.

a detailed time-course study on the transcriptional effects of TGF/β, we have observed a 35% decrease in PRL gene transcription 6 h after TGF/β treatment (data not shown).

TGF/β Does not Affect Pit-1 Gene Expression
One mechanism by which TGF/β might inhibit PRL gene transcription is via inhibition of Pit-1 gene expression. Pit-1 is a pituitary-specific transcription factor regulating the expression of PRL and GH genes (38, 39). In a separate experiment, we compared the effects of TGF/β on PRL mRNA and Pit-1. As shown in Fig. 4A, Ca²⁺ increased PRL mRNA by about 2-fold over that in SFM controls after 24 h. TGF/β completely blunted this effect of Ca²⁺. In contrast, the presence of Ca²⁺ or Ca²⁺ plus TGF/β had no effect on Pit-1 mRNA. These findings are supported by the Northern hybridization data shown in Fig. 2B. In this experiment, the presence of Ca²⁺ lowered Pit-1 mRNA levels, although correction for loading/transfer based on GRP78 mRNA levels indicate that the effect of Ca²⁺ on Pit-1 mRNA is slight. More importantly, TGF/β had no effect on Pit-1 mRNA levels. Additionally, the effect of TGF/β on Pit-1 gene transcription was assessed by run-on assay, using labeled RNAs from the experiment shown in Fig. 2. While TGF/β decreased PRL gene transcription by about 50% (Fig. 2, A and C), it had no effect on Pit-1 gene transcription (Fig. 4B). Pit-1 gene transcription approximately doubled in the presence of Ca²⁺, regardless of other treatments.

DISCUSSION
Our data demonstrate that TGF/β is a potent and specific inhibitor of both basal and Ca²⁺-stimulated PRL mRNA expression in GH3 cells. The inhibitory effect of TGF/β occurred without changes in the expression of several constitutively expressed genes (histone-3 and GRP78) or in the levels of GH and Pit-1 mRNAs. Ramsdell (20) has also reported that TGF/β and activin inhibit PRL production by GH3 cells. This implies that inhibition of PRL gene expression is a physiological effect of TGF/β or a closely related factor.

The results of run-on transcription assays were striking, in that Ca²⁺ increased PRL gene transcription by only 30%, yet it induced a 7-fold increase in PRL mRNA levels. This confirms our previous findings (7) and supports the idea that Ca²⁺ induction of PRL gene expres-
sion has a substantial posttranscriptional component. The inhibition of PRL gene expression by TGFβ, on the other hand, appears to occur mainly at the transcriptional level. PRL gene transcription and mRNA levels decreased 50-60% in TGFβ-treated cells. Additionally, nuclear PRL precursor RNAs in TGFβ-treated cells decreased proportionately with the mature PRL mRNA.

Our data indicate that TGFβ does not act on PRL gene expression via an inhibition of Pit-1 gene expression. These data do not address the possibility of TGFβ-induced posttranslational modifications of the Pit-1 protein. Alternatively, Kerr et al. (40) provided evidence that TGFβ inhibits transin/stromelysin gene transcription through a TGFβ inhibitory element (TIE), GAGTTGGGGA. This element acts as an AP-1 site, and activation of c-fos is required for TGFβ repression of transin/stromelysin gene transcription (40). These researchers also noted that apparent TIEs with the consensus sequence GnnTGTTGa reside within the promoters of the other TGFβ-inhibited genes, including proliferin. Although the activities of these TIEs have yet to be determined, it is interesting to note that two putative TIEs reside within the rat PRL gene promoter. These are GAATTGGGA at position -1016 and GCTTTGGGGT at position -1561 relative to the transcription start site. We have observed an increase in c-fos mRNA in response to TGFβ in GH3 cells (our unpublished observations). However, since c-fos mRNA is also readily detectable in GH3 cells cultured in SFM plus Ca2+ (our unpublished observations), and since c-fos mRNA is increased in GH3 cells by other treatments that are associated with an increase in PRL mRNA (41), any contribution of c-fos to the effect of TGFβ is likely to be complex. The AP-1 family represents a large number of proteins, of which some are stimulatory and some inhibitory at AP-1 sites (42). Thus, it is possible that another AP-1 protein gene is induced by TGFβ which heterodimerizes with c-fos to inhibit the expression of specific genes. Further experiments are needed to examine whether c-fos is required for the effects of TGFβ on PRL gene transcription and whether the putative TIEs within the PRL promoter confer transcriptional repression by TGFβ.

The potency and specificity of the inhibition of PRL gene expression by TGFβ in our studies and those of Ramsdell (20) suggest that there may be a physiological role for TGFβ in regulation of lactotroph function. Expression of the PRL and GH genes is dependent on the presence of Pit-1/GHF-1 (38, 39, 43). However, the heterogeneity of PRL-producing cells in the pituitary (44) and in tumor lines (45) makes it likely that the specification of cell type also requires additional factors. It is possible that factors, such as TGFβ, that regulate both PRL gene expression and lactotroph proliferation may be involved in this specification of pituitary cell phenotype.

MATERIALS AND METHODS

Cell Culture

GH3 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in suspension culture (46). The 235–1 cells were generously provided by Dr. H. Samuels (New York University School of Medicine) and maintained in Spinner culture medium (46) containing 10% iron-supplemented calf serum (HyClone, Logan, UT). For experiments, cells were spun out of growth medium, washed once in SFM (46), and plated onto 35- or 60-mm tissue culture dishes (Falcon, Oxnard, CA). Treatments were added at the time of plating, and the cells were cultured for 18–24 h, as indicated, in a 37 C humidified incubator at 2% CO2.

TGFβ and Dex

Porcine TGFβ1 and TGFβ2 (R & D Systems, Minneapolis, MN) or human TGFβ1 (Collaborative Research, Bedford, MA) were resuspended at a concentration of 2 ng/ml in sterile diluent (4 mM HCl and 1 mg/ml BSA) and added to GH3 cell cultures at final concentrations ranging from 0.02–10 ng/ml. Diluent alone had no effect on any of the mRNAs measured. Dex (Sigma, St. Louis, MO) was dissolved in PBS at 50 mM and used at a final concentration of 100 nm.

Run-On Transcription Assay

GH3 cells (five 100-mm dishes per group) were cultured for 20 h in SFM alone or in the presence of 0.5 mM CaCl2 (Ca2+), Ca2+ plus 5 ng/ml TGFβ, or Ca2+ plus 100 mM Dex. Cells were collected by scraping, pooled, and washed once in ice-cold PBS, and then nuclei were isolated and used for run-on transcription, as previously described (7), using 300 mCi [32P]UTP (650 Ci/mmol; ICN, Irvine, CA)/reaction. Labeled run-on RNA (final volume, 0.4 ml) was isolated essentially as previously described (47). The final RNA pellet was washed with 70% ethanol, drained dry, and resuspended in 200 μl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.02% sodium dodecyl sulfate). The RNA was denatured by the addition of 50 μl 1 M NaOH and incubation on ice for 15 min, and then neutralized by the addition of 100 μl 1 M HEPES-free acid plus 650 μl TES buffer. The total counts per min of 32P-labeled RNA in 5 μl of each sample were determined, and equal counts per min in 1 ml TES buffer were added to 1 ml TES buffer plus 0.5 mM NaCl, then hybridized to DNA dots on nitrocellulose filters that were prehybridized as previously described (7). DNA dots were made with linearized and denatured plasmids containing either no insert (Bluescript KS+), a PRL cDNA clone (PRL-SK+; see below), or a plasmid containing a Pit-1 cDNA (see below). Hybridization was carried out at 52 C for 2 days. Filters were washed twice in 2 X SSC (0.3 mM NaCl and 0.3 mM sodium citrate, pH 7) for 1 h at 65 C, then treated with 1 mg/ml RNase-A (Boehringer Mannheim, Indianapolis, IN) in 2 X SSC for 30 min at 37 C. After a final wash in 2 X SSC at 37 C for 1 h, filters were blotted dry and analyzed using a Betascope 603 blot analyzer (Betagen, Waltham, MA).

RNA Isolation and Analysis

Cytoplasmic RNA was prepared from cell lysates after removal of nuclei by centrifugation. Cytoplasmic lysates (5 ml) were combined with an equal volume of 2 X proteinase-K buffer [2 x 0.2 M Tris-Cl (pH 7.5), 0.44 mM NaCl, 2% sodium dodecyl sulfate, and 25 mM EDTA] plus 200 μg/ml proteinase-K and incubated for 30 min at 37 C. The samples were extracted once with phenol-chloroform (1:1), twice with chloroform, and then made 0.25 M in NaCl and ethanol precipitated. Ten-microgram aliquots of RNA were electrophoresed on 1% agarose-formaldehyde gels and analyzed by Northern blot hybridization (48). Nuclear RNA was isolated from GH3 cells (five 100-mm dishes per group) that were cultured in SFM plus Ca2+ in the presence and absence of 5 ng/ml TGFβ for 24 h. Cells were collected by scraping, pooled, washed once in ice-cold PBS, and then resuspended in 1.8 ml PBS. Cells were lysed by the
addition of 0.2 ml Nonidet P-40 (5% in PBS), incubated on ice for 3 min, then centrifuged at 1000 x g for 5 min at 4 C. Supernatants were used for preparation of cytoplasmic RNA, as described above. The nuclear RNA was isolated essentially as previously described (49). Ten-microgram aliquots of nuclear RNA were electrophoresed through 1% agarose-formaldehyde gels, then transferred to nitrocellulose for Northern blot hybridization analysis.

RNA was also measured by cytoplasmic dot hybridization, as previously described (50). The PRL GH, GRP78, and histone-3 DNA probes used in this study have been previously described (7), except that the PRL cDNA used in this study was the insert from pPRL-1 (7), which was reinserted into the PstI site of the Bluescript SK+ vector (Stratagene, La Jolla, CA). The PRL probe used for Northern blot hybridization analysis of nuclear RNA was generated by polymerase chain reaction using oligonucleotide primers to exons 1 and 5 of the rat PRL gene, as previously described (49). Ten-microgram aliquots of nuclear RNA were electrophoresed through 1% agarose-formaldehyde gels, then transferred to nitrocellulose for Northern blot hybridization analysis.

Acknowledgments

We thank Drs. Eileen Roark and Robert Kosher for their advice on the use of TGFβ and for provision of samples of TGFβ1 and TGFβ2. We thank Melissa Lail for her expert technical assistance.


Address requests for reprints to: Dr. Bruce A. White, Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06030.

This work was supported by NIH Research Grant DK-43064.

REFERENCES

1. Lamberts SWJ, Macleod RM 1990 Regulation of prolactin secretion at the level of the lactotroph. Physiol Rev 70:279–318
10. Somasekhar MB, Gorski J 1988 Two elements of the rat prolactin 5' flanking region are required for its regulation by estrogen and glucocorticoids. Gene 69:13–21
11. Potter E, Nicolaisen AK, Evans RM, Rosenfeld MG 1981 Thyrotropin-releasing hormone exerts rapid nuclear effects to increase production of the primary prolactin mRNA transcript. Proc Natl Acad Sci USA 78:6662–6666