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The International Journal of Biochemistry & Cell Biology 34 (2002) 1571–1585

IJBCB

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Antagonism of glucocorticoid receptor transactivity and cell growth inhibition by transforming growth factor-β through AP-1-mediated transcriptional repression

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Received in revised form 5 March 2002; accepted 26 March 2002

Abstract

We have examined the interaction of the glucocorticoid receptor (GR) and transforming growth factor-β (TGF-β) signal pathways because of their mutual involvement in the regulation of cell growth, development and differentiation. Most studies of this cross-talk event have focused on the effects of glucocorticoids (GCs) on TGF-β responses. In this work, we show that TGF-β can antagonize dexamethasone (Dex)-mediated growth suppression in mouse fibrosarcoma L929 cells. TGF-β also repressed GR-mediated reporter (pMMTV-CAT) gene expression in a concentration-dependent manner, with an IC_{50} of 5 ng/ml of TGF-β. Maximal inhibition (76%) was observed at 10 ng/ml of TGF-β. Conversely, Dex inhibited TGF-β-mediated promoter (p3TP-Lux) activity in these same cells. As TGF-β inhibition of GR-mediated gene expression occurred after Dex-mediated nuclear translocation of GR, we conclude that TGF-β inhibition of GR signaling occurs at the level of GR-mediated transcription activity. However, TGF-β did not repress GR-mediated gene expression using the $pGRE_2E1B$-CAT minimal promoter construct, suggesting that TGF-β did not inhibit intrinsic GR activity but, rather, required DNA-binding factor(s) distinct from GR. As the *MMTV* promoter contains several putative AP-1 binding sites, we hypothesized that AP-1, a transcription factor composed of c-jun and c-fos proteins, might be involved in the TGF-β inhibition of GR functions. Curcumin, a potent inhibitor of AP-1 expression, completely abolished the inhibitory effect of TGF-β on GR-mediated gene expression without affecting GR activity in the absence of TGF-β, and this drug blocked TGF-β-induced binding of AP-1 to a response element derived from the *MMTV* sequence. Furthermore, curcumin abolished TGF-β inhibition of Dex-induced growth suppression. Taken as a whole, our data suggest that TGF-β can antagonize the growth inhibitory properties of GR by blocking GR transactivity at complex promoters through a mechanism involving transcriptional repression by DNA-bound AP-1.
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Keywords: Steroid receptor; Glucocorticoid; Transforming growth factor-β; AP-1; Cell growth

1. Introduction

Cross-talk between signaling pathways enables the multiplication of a cell's regulatory potential and provides diversity to cell-specific responses. In the case of signaling by the glucocorticoid receptor (GR), the accumulating evidence indicates that a variety of effector molecules from previously unrelated signaling pathways can have profound effects on the magnitude of a cell's response to glucocorticoids (GCs). For example, GR-regulated transcription can be modulated by oncogenes such as ras (a GTP-binding

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PII: S1357-2725(02)00057-2

protein) and mos (a cytoplasmic protein kinase), and c-Fos and c-Jun, which bind to form the activator protein-1 (AP-1) transcription factor [1,2]. Regulation of GR responses by growth factors controlling tyrosine kinase cascades (e.g. TGF-α and EGF), or serine/threonine kinase cascades (e.g. TGF-β) have also been reported [3–6]. In the case of TGF-β, reciprocal control between this growth factor and GCs has been demonstrated in a variety of biological processes in fibroblast [5], osteoblast [6,7] and epithelial cells [8] through modulating the expression of target genes. These findings demonstrate that an elaborate coupling of the GR signaling pathway with growth factor signaling mechanisms must exist, perhaps to impose an additional layer of control on hormone- and growth factor-mediated responses.

Glucocorticoid hormones regulate a wide range of biological processes, such as cell proliferation, inflammation, and immunity, by interacting with the cognate intracellular GR. The GR is a ligand-dependent transcription factor belonging to the superfamily of steroid/thyroid hormone receptors [9]. Although many of the details of GR activation are not known, it is generally accepted that the first event in this process is the binding of hormone to the untransformed GR, present as a cytoplasmic, heteromeric complex containing heat shock proteins (hsps), such as hsp90 and hsp56 [10,11]. Upon hormone binding, the GR dissociates from the hsps and the hormone-bound GR translocates to the nucleus [12]. In the nucleus, the hormone-receptor complex (the transformed GR) acts as a transcription factor and binds to specific DNA sequences called glucocorticoid response elements (GREs). Depending on the nature of the GRE, GR binding can result in activation or repression of genes containing GR-binding sites. Alternatively, GR can also modulate the expression of genes through a GRE-independent mechanism, such as protein-protein interactions of GR with other sequence-specific DNA binding factors or coactivators. GR and two groups of physiologically important transcription factors, AP-1 and nuclear factor-kB (NF-kB), have been reported to mutually interfere with each other's activity [13–15]. On the other hand, studies have supported a positive role for the AP-1 transcription factor in regulating GR gene expression in certain fibroblast cells [16]. However, the cross-talk between GR and AP-1 is a more complex process, and the outcome is determined by the composition of the AP-1 subunits, alterations in local DNA topology and relative positions of GRE and AP-1 sites within the promoter [17,18]. Curcumin, a yellow ingredient from turmeric, is known to inhibit AP-1 expression in mouse fibroblasts [19–21].

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that plays an important role in the regulation of cell growth, development, differentiation, ECM formation, inflammation and immune function in many biological systems (for reviews see [22]). TGF-β exerts both positive and negative effects on cell growth, the final effect depending on a number of factors, such as cell type, growth conditions, stage of differentiation, and the presence of other growth regulators [23]. TGF-β is a potent growth inhibitor for most epithelial cells in culture. However, TGF-β has been widely viewed as a growth stimulator for mesenchymal cells [24,25]. Similarly, a proliferative role of TGF-β has also been reported in epithelial cells [26]. Several studies have been shown to support a role for TGF-β in the promotion of tumorigenicity in vivo [27,28]. The biological effects of TGF-β are mediated through two types of transmembrane serine/threonine kinase receptors (type I and type II), and the highly conserved Smad family of proteins has been identified as intracellular signal transducers to relay the TGF-β signal to the nucleus [29,30]. One of the first cellular responses to TGF-β is the rapid transcriptional activation of a set of specific genes, some of which are genes encoding transcription factors such as AP-1 and NF-kB [31,32]. AP-1 and NF-kB are important transcription factors in that they play a critical role in the regulation of cell growth, inflammation and immunity [14,15].

TGF-β and glucocorticoid signaling pathways interact both positively and negatively in regulating a variety of physiological and pathological processes. TGF-β and GCs can induce growth arrest in human monocytoid leukemic, prostate and osteosarcoma cells [33–35]. On the other hand, glucocorticoid and TGF-β antagonistically regulate bone formation [6] and tight junction activity [8]. Furthermore, GCs inhibit the TGF-β induced expression of extracellular matrix proteins including fibronectin [36], collagen [37], and proteinase inhibitors, such as certain inhibitors of metalloproteinases [38]. Hence, GCs and TGF-β may be considered as important regulators in the homeostatic control of cell growth, wound healing

and fibrosis. But, the molecular mechanisms involved in the cross-talk between TGF-β and glucocorticoid signaling pathways remain to be established. Although it has been recently demonstrated that GCs inhibit the expression of TGF-β type 1 receptor by decreasing type 1 receptor promoter activity [6,7] and that GCs repress TGF-β-induced activation of plasminogen activator inhibitor-1 gene transcription by directly interacting with the Smad 3 protein [39], little evidence exists as to the molecular mechanisms by which TGF-β regulates the GR signaling pathway.

In this work, we investigate the molecular mechanisms by which TGF-β inhibits hormone-induced GR-mediated gene expression and cell proliferation in murine fibrosarcoma L929 cells. The results reported here show that TGF-β inhibition of GR-mediated gene expression occurs after GR has translocated to the nucleus, indicating that TGF-β inhibition of GR signaling occurs at the level of GR-mediated transcription. However, TGF-β did not repress GR-mediated gene expression as measured by a minimal promoter containing only GREs, suggesting that the TGF-β inhibition of GR signaling requires additional DNA-binding sites or factors distinct from GREs or the GR itself. Curcumin, a potent inhibitor of AP-1 activity, totally abrogates the inhibitory effect of TGF-β on GR-mediated gene expression without affecting GR activity in the absence of TGF-β. In addition, curcumin abolishes TGF-β inhibition of dexamethasone (Dex)-induced growth suppression. Moreover, the inhibition of GR-mediated gene expression by TGF-β is associated with increased c-Jun and c-Fos binding to AP-1 sites and binding by AP-1 is inhibited by curcumin. These results demonstrate that antagonism of GR by TGF-β occurs by a mechanism involving transcriptional repression by the AP-1 transcription factor.

2. Materials and methods

2.1. Growth inhibition assay

Mouse fibrosarcoma L929 cells (5×10^3 cells/well) were plated in 96-well culture plates and allowed to adhere to substrate for 24 h in DMEM containing 10% bovine calf serum that had been treated with dextran-coated charcoal (DCC). The DCC serum concentration was then reduced to 1%, and the cells were incubated for 7 days in the presence of Dex (10 and 100 nM), TGF-β_1 (5 ng/ml), curcumin (1.0 μM), and a combination of all three with medium changes every 2 days. At the end of the incubation, a calorimetric assay (MTT) utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to determine relative cell numbers [40]. Briefly, 0.1 mg (50 μl of 2 mg/ml) of MTT was added to each well and incubated at 37 °C for 2 h. The MTT medium was then removed, and 150 μl of DMSO was added to each well and allowed to shake gently for 15 min at room temperature. Absorbance was measured at 595 nm using a Molecular Devices SOFTmax microtiter plate reader.

2.2. Chloramphenicol acetyl transferase (CAT) assay

L929 cells were co-transfected with pSV2neo and a two-fold excess of pMMTV-CAT (LMCAT cells), pGRE$_2$E1B-CAT (LGRE cells) or pCAT-control (LSVCAT cells), using lipofectin as the carrier as described previously [41]. This was followed by selection for stably-transfected, cloned cell lines using G418 antibiotic at 0.4 mg/ml. Once established, all cell lines were grown in an atmosphere of 5% CO_2 at 37 °C in DMEM containing 0.2 mg/ml G418 and 10% DCC bovine calf serum. Measurement of CAT enzyme activity was performed according to the method of Nordeen et al. [42] with minor modifications. Briefly, cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5) and centrifugation at $14,000 \times g$ for 10 min. Aliquots of lysate containing equal protein content were added to an enzymatic reaction mixture containing acetyl CoA synthetase, [^{3}H]sodium acetate, coenzyme A (CoA) and ATP. Radioactively-labeled acetyl CoA was first generated enzymatically from CoA and labeled acetate. Acetylation of chloramphenicol was then initiated by adding cell lysate containing the CAT enzyme. The reaction was stopped by extraction with cold benzene, and 75% of the organic phase was taken and counted.

2.3. Transient transfection and luciferase reporter assays

The TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter construct (p3TP-Lux) was used to determine whether Dex mutually

antagonizes TGF-β responsive promoter activity [43]. L929 cells were plated at a density of 2×10^5 in $25\,cm^2$ culture flasks. At 80–90% confluence, the cells were transfected with a total of 25 μg of DNA by use of 30 μg of lipofectamine and incubated for 5 h at 37 °C in HEPES-buffered saline. A β-galactosidase plasmid was co-transfected as an internal control to normalize for variations in transfection efficiency. The cells were fed 5 h following transfection. Twenty-four hours after transfection, the DMEM medium containing 10% DCC serum was replaced with DMEM with 1% DCC serum for 6 h, and then serum-free DMEM. Forty-eight hours after transfection, cells were treated first with Dex (1.0 μM) for 3 h, followed by TGF-β_1 (5 ng/ml) for an additional 20 h. The cell extracts were prepared by use of reporter lysis buffer (Promega) and the luciferase activity was measured using a luminometer, and the data was expressed as relative units after normalization to β-galactosidase activity.

2.4. Preparation of nuclear protein extracts and EMSA assay

Confluent LMCAT cells in 10 cm diameter dishes were treated with and without curcumin (1 μM) in the absence and presence of TGF-β_1 (5 ng/ml). After 3 h, the nuclear proteins were essentially prepared as described by Dignam et al. [44]. In brief, the treated and control cells were scraped into PBS, and suspended in pellet lysis buffer (10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, and 0.5% Nonidet-P40). The nuclei were separated from the cytosol by centrifugation at $3000 \times g$ for 15 min. Then the nuclei were treated with buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and centrifuged at $3000 \times g$ for 15 min. The nuclear pellets were further treated by stirring for 60 min at 4 °C in buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.42 M NaCl, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Nuclear extracts were obtained by centrifugation for 60 min at $25{,}000 \times g$ and demineralized through a Sephadex G-25 column equilibrated with buffer C (HEPES 5 mM, pH 7.9, 0.02 M KCl, 0.04 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF).

Electrophoretic mobility shift assays (EMSA) were performed as previously described by Takeshita et al. [31] with some modifications. Double-stranded oligonucleotides representing the AP-1 and TGF-β response element (TβRE) sites within the *MMTV* promoter were custom designed and obtained from Genosys. The *MMTV* AP-1 and TβRE sequences used in the gel shift assays were as follows: 5′-TGT,TAA, GAA,ATG,AAT,CAT,TAT,CTT,TTA,GTA-3′ (AP-1 sequence) and 5′-GAT,GTG,AGA,CAA,GTG,GTT, TCC,TGA,CTT,GGT-3′ (TβRE sequence). The AP-1 and TβRE oligonucleotides were end-labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase. The labeled oligonucleotides were purified using probe Quant^{TM} G-50 microcolumns. Binding reactions were performed using 2.5 μg of nuclear protein, buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 12% glycerol (v/v), 1 mM DTT, 0.5 mM PMSF), 2 μg of Poly (dI-dC), and 30,000 cpm of ^{32}P-labeled oligonucleotides in a final volume of 25 μl. Poly (dI-dC) and nuclear extract were first incubated at 4 °C for 10 min before addition of the labeled oligonucleotide. Reaction mixtures for the binding were incubated for an additional 15 min at 4 °C after addition of the labeled oligonucleotide. Unlabeled double-stranded oligonucleotides were used as the competitors. To confirm that protein-DNA complexes contained AP-1, gel shift assays were carried out by incubating the nuclear extract with c-Jun (Santa Cruz 45X, rabbit polyclonal specific for c-Jun) and c-Fos (Santa Cruz 52X, rabbit polyclonal specific for c-Fos) antibodies for 15 min at 4 °C prior to the addition of ^{32}P-labeled oligonucleotides. DNA-protein complexes were electrophoresed on nondenaturing 4% polyacrylamide gel at 170 V for 2.0 h in $0.5 \times$ TBE buffer (44 mM Tris, 44 mM boric acid, and 1 mM EDTA (pH 8.0)). Gels were vacuumed, dried, and exposed to Kodak X-ray film at −70 °C.

3. Results

3.1. TGF-β inhibits Dex-induced growth suppression

Experiments were carried out to determine the molecular mechanisms by which TGF-β regulates the GR signaling pathway in mouse fibrosarcoma L929 cells. Fig. 1 shows the effect of TGF-β on Dex-induced growth suppression in mouse fibroblast L929 cells. The results show that treatment of L929 cells with various concentrations of Dex (1–100 nM) resulted in concentration-dependent growth inhibition (Fig. 1B).

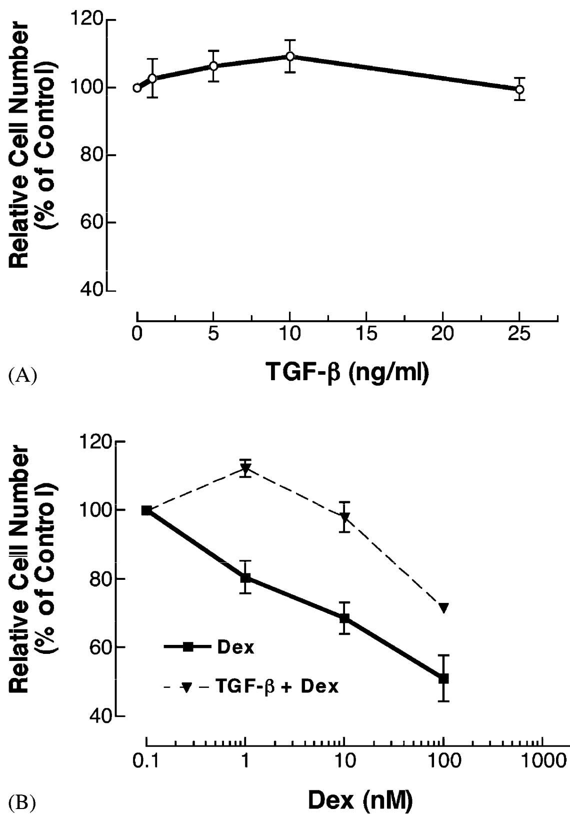

Fig. 1. TGF-β reverses Dex-induced growth suppression of L929 cells. (A) L929 cells (5×10^3 cells/well) grown in reduced serum (1%) were incubated for 7 days in the presence of increasing concentrations of TGF-β_1, followed by measurement of relative cell number by MTT colorimetric assay; (B) same as above, except cells were exposed to increasing concentrations of Dex in the presence or absence of TGF-β_1 (5 ng/ml). The data of panels A and B are representative of three independent experiments. In each experiment, cell densities in three replicate wells were measured per condition. Thus, each value corresponds to the mean ± S.E.M. of nine wells, with 100% growth representing untreated control cells.

Fifty percent inhibition was observed at 100 nM of Dex. In contrast, TGF-β alone showed no effect on cell proliferation up to 25 ng/ml (Fig. 1A). However, in the presence of TGF-β (5 ng/ml), Dex-induced growth inhibition was blocked or reduced at all concentrations of hormone (Fig. 1B). These data confirm the previously reported growth inhibitory effect of Dex on mouse L929 fibroblasts [45], and further indicate that TGF-β can antagonize glucocorticoid-induced growth suppression in these cells.

3.2. *Dexamethasone inhibits TGF-β-responsive promoter activity*

To determine whether Dex can mutually antagonize TGF-β-mediated responses in these cells, TGF-β-dependent promoter activity was analyzed using the TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter construct (p3TP-Lux). p3TP-Lux contains three consecutive TPA (tetradecanoylphorbol acetate) response elements and a portion of the plasminogen activator inhibitor gene promoter linked to the luciferase gene [43]. In the experiments of Fig. 2A, L929 cells were transiently transfected with p3TPLux in order to assess the effects of various combinations of TGF-β and Dex. A β-galactosidase plasmid was co-transfected to monitor the transfection efficiency. Forty-eight hours after transfection, cells were treated first with Dex (1.0 μM) for 3 h, followed by TGF-β (5 ng/ml) for an additional 20 h. Cells were harvested, and luciferase activity was measured using a luminometer and was expressed as relative units after normalization to β-galactosidase activity. TGF-β treatment showed a 3.2-fold increase in luciferase activity compared to control. On the other hand, Dex (1.0 μM) showed no effect on control cells, but completely inhibited TGF-β-stimulated luciferase activity. These data confirm that glucocorticoid agonists can repress TGF-β activity in our cells, as has been reported in other cell lines and tissues [6,8,39].

3.3. *TGF-β inhibits GR-mediated gene expression in LMCAT cells*

Because little is known with respect to the mechanism by which GR signaling is inhibited by TGF-β, we set out to uncover this mechanism. To determine the molecular mechanisms by which TGF-β inhibited glucocorticoid-mediated growth suppression, we tested the effects of TGF-β on Dex-induced GR-mediated gene expression in LMCAT cells. The LMCAT cells were obtained by stably transfecting L929 cells with the pSV2neo and pMMTV-CAT plasmids [41]. The pMMTV-CAT construct is a glucocorticoid-inducible reporter plasmid, containing the complete mouse mammary tumor virus-long terminal repeat (*MMTV-LTR*) promoter, upstream of chloramphenicol acetyltransferase (*CAT*). The *MMTV* promoter has been used extensively to study

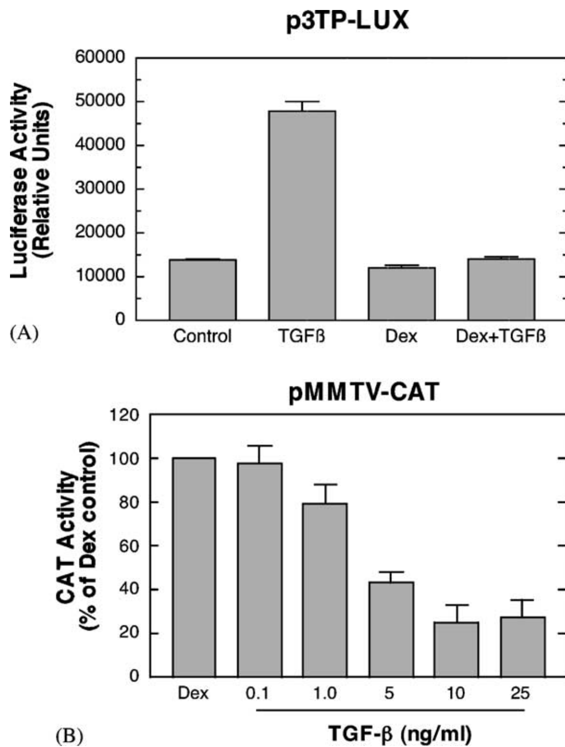

Fig. 2. Mutual antagonism of glucocorticoid and transforming growth factor-β signaling. (A) Dex inhibits TGF-β-responsive promoter activity in L929 cells. Cells were transiently co-transfected with p3TP-LUX and β-galactosidase plasmids. Forty-eight hours after transfection, cells were treated with various combinations of Dex (1.0 μM) and TGF-β_1 (5 ng/ml), as indicated. Luciferase activity was measured and expressed as relative units after normalization to β-galactosidase activity. The results shown represent mean ± S.E.M. of two independent experiments. Control: no treatment. TGF-β: TGF-β for 20 h. Dex: Dex for 23 h. Dex + TGF-β: Dex for 3 h, followed by TGF-β for an additional 20 h; (B) TGF-β inhibits GR-mediated gene expression. L929 cells stably transfected with the pMMTV-CAT reporter (LMCAT cells) were pretreated with TGF-β_1 (0.1–25 ng/ml) for 3 h, followed by incubation with Dex (100 nM) for an additional 20 h. Cell lysates were prepared and assayed for CAT enzyme activity. The data shown represent the mean ± S.E.M. of three to six independent experiments.

GR-mediated gene expression in a number of transformed and untransformed cell lines of mesenchymal and epithelial origins. GCs induce transcription by the *MMTV* promoter upon binding to four GRE sites located between −202 and −59 bases upstream of the transcription start site [46]. As shown in Fig. 2B, TGF-β inhibited Dex-induced *CAT* gene expression in a concentration-dependent manner, and an IC_{50} was observed at a concentration of 5 ng/ml of TGF-β. Maximal inhibition (76%) was observed at 10 ng/ml of TGF-β. However, TGF-β alone had no effect on basal *MMTV-CAT* gene expression (data not shown). Taken together, the results of Fig. 2 show that mutual, antagonistic cross-talk between the GR and TGF-β signaling pathways exists in the mouse L929 cell line.

3.4. TGF-β has no effect on CAT gene expression in LSVCAT cells

To test the possibility that TGF-β inhibition of GR-mediated gene expression was the result of a general effect on gene transcription or post-transcriptional modification of CAT expression, we measured the effect of TGF-β on *CAT* gene expression in LSVCAT cells made by stably transfecting L929 cells with the pCAT-Control plasmid. In pCAT-Control, expression of CAT is under the constitutive control of the Simian Virus 40 (SV40) early promoter. The results of these experiments are shown in Fig. 3. There was no reduction in *CAT* gene expression in response to various concentrations of TGF-β in the presence of 100 nM

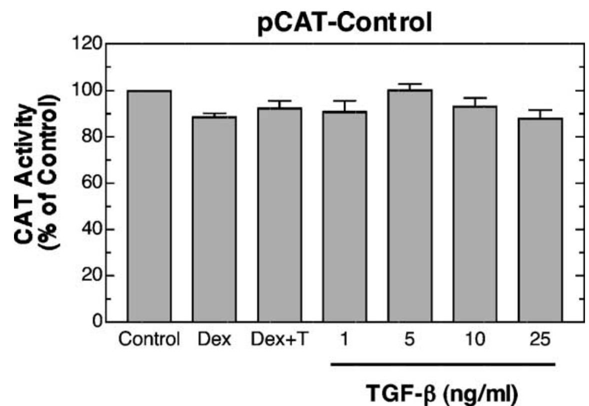

Fig. 3. TGF-β has no effect on CAT expression controlled by the SV40 promoter. L929 cells (LSVCAT) stably transfected with pCAT-Control (SV40 promoter) were treated with various combinations of Dex and TGF-β_1, as indicated with TGF-β_1 (1–25 ng/ml) for 3 h, followed by incubation with Dex (100 nM) for an additional 20 h, and then the cell lysates were prepared and assayed for CAT enzyme activity. The data shown represent the mean ± S.E.M. of three to six independent experiments. Control: no treatment. Dex: Dex (100 nM) for 20 h. Dex + T: Dex (100 nM) plus TGF-β_1 (25 ng/ml) for 20 h. TGF-β: TGF-β_1 for 20 h at the indicated concentration.

Dex. This suggests that the actions of TGF-β on GR-induced *CAT* gene expression in LMCAT cells, are not the result of a non-specific decrease in general gene transcription or of alterations in CAT mRNA or protein stability. Moreover, no gross alterations in morphology were observed in these cells in response to growth factor treatment.

3.5. TGF-β inhibits GR-mediated gene expression after nuclear translocation of GR

To determine the overall stage of GR signaling affected by TGF-β, LMCAT cells were pretreated with 100 nM Dex for 3 h before addition of TGF-β. For comparison, we also treated LMCAT cells with TGF-β for 3 h before addition of 100 nM Dex. The 3 h period for treatment with Dex was selected in these experiments since we have previously shown that a 1 h treatment of LMCAT cells with 1 μM Dex resulted in near-complete nuclear translocation of GR [41]. As shown in Fig. 4, TGF-β followed by Dex treatment resulted in marked inhibition of GR-mediated gene expression (58 and 72% inhibition at 5 and 10 ng/ml TGF-β, respectively). However, Dex treatment followed by TGF-β also yielded a similar level of inhibition (69 and 65% at 5 and 10 ng/ml TGF-β, respectively), indicating that TGF-β inhibition of GR-mediated gene expression most likely occurs at the level of transcription from the *MMTV* promoter. It is possible that TGF-β treatment of LMCAT cells can somehow promote GR turnover resulting in a decrease in GR protein levels. To test this possibility, Western immunoblot analysis of GR protein was carried out using cytosolic and nuclear pellet fractions derived from control and TGF-β treated cells. It was found that GR protein levels in cytosolic and nuclear pellet fractions derived from TGF-β treated cells were unchanged compared to control cells (data not shown), indicating that TGF-β inhibition of the GR activity is not due to the loss of GR protein. Furthermore, these data indicate that TGF-β does not alter the subcellular localization of GR.

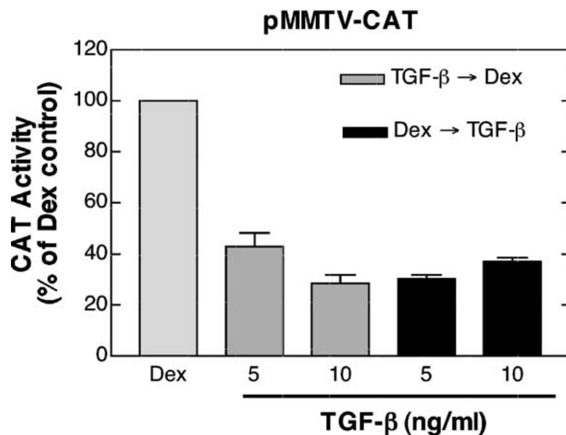

Fig. 4. TGF-β inhibition of GR occurs after hormonal activation of the receptor. LMCAT cells were treated with 100 nM Dex for 3 h before addition of TGF-β_1 (5 and 10 ng/ml) and subsequent incubation for 20 h (Dex → TGF-β). LMCAT cells were also treated with TGF-β for 3 h before addition of 100 nM Dex and subsequent incubation for 20 h (TGF-β → Dex). Lysates were prepared and assayed for CAT enzyme activity. The results shown represent mean ± S.E.M. of two independent experiments.

3.6. TGF-β inhibition of GR requires transcription factors other than GR

The *MMTV* promoter employed in the LMCAT cells is approximately 1.2 kb in size and it contains, in addition to GREs, critical binding sites for a variety of widely-expressed transcription factors, including AP1, NF-1, Oct-1 and Sp1 [47,48]. Therefore, we could not exclude the possibility that the actions of TGF-β occurred via a DNA-binding factor(s) that could inhibit or counteract the transcription enhancement function of GR. To test this possibility, L929 cells stably transfected with the pGRE$_2$E1B-CAT minimal promoter (LGRECAT cells) were subjected to TGF-β treatment in the presence and absence of Dex. In the pGRE$_2$E1B promoter, expression of CAT is controlled by two tandemly-linked, synthetic GREs and a TATA box [49]. In the experiments of Fig. 5, LMCAT and LGRECAT cells were pretreated with 100 nM Dex for 3 h before addition of TGF-β. As expected, Dex followed by TGF-β treatment resulted in marked inhibition of GR-mediated gene expression in LMCAT cells (Fig. 5A). In contrast, no inhibition of GR-mediated gene expression by TGF-β was observed in LGRECAT cells (Fig. 5B). These data suggest that TGF-β inhibited GR-mediated gene transcription in LMCAT cells via activation or inhibition of transcription factor(s) distinct from GR. Furthermore, these findings indicate that the TGF-β inhibitory effect on GR-mediated gene expression was

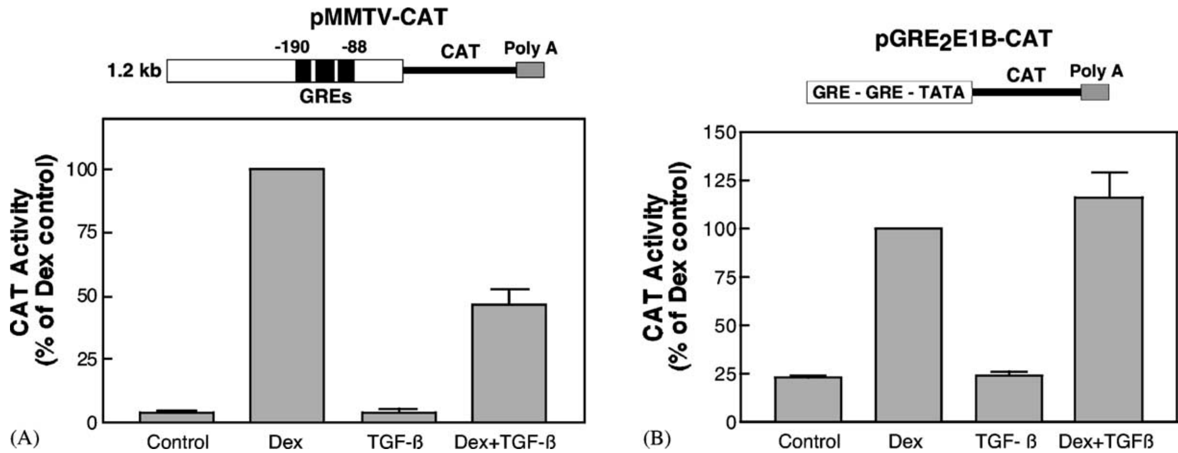

Fig. 5. TGF-β does not inhibit GR-mediated *CAT* gene expression from a minimal promoter. L929 cells stably transfected with the complex pMMTV-CAT reporter (panel A) or with the pGRE$_2$E1B-CAT minimal reporter (panel B) were exposed to various combinations of Dex and TGF-β_1, as indicated. Lysates were prepared and assayed for CAT enzyme activity. The results shown represent the mean ± S.E.M. of three independent experiments. Control: no treatment. Dex: Dex (100 nM) for 20 h. TGF-β: TGF-β_1 (5 ng/ml) for 20 h. Dex + TGF-β: TGF-β (5 ng/ml) for 3 h, followed by incubation with Dex (100 nM) for an additional 20 h.

not due to an inhibition of intrinsic GR activity, either by direct competition for DNA-binding sites (GREs) or via protein–protein interaction with some inhibitor.

3.7. Curcumin, an inhibitor of AP-1, abolishes TGF-β inhibition of GR-mediated CAT gene expression

As shown in Fig. 6, the *MMTV* promoter contains binding sites for a variety of transcription factors. Of particular interest to us was the presence of four AP-1 sites at −4, −723, −749, and −769 bp, as well as a TGF-β response element (TβRE) located at −139 bp. Because it had been demonstrated that TGF-β_1 is a potent inducer and activator of AP-1 [50], and because mutual antagonism of GR and AP-1 has been reported [13,14], we reasoned that AP-1 might be involved in TGF-β inhibition of GR function. However, in most cases, repression of GR by AP-1 has been shown to result from a direct interaction between these two factors [14]. Given that TGF-β did not inhibit GR activity from the pGRE$_2$E1B-CAT reporter (Fig. 5B), the latter mechanism was not likely to be operating in our system. Instead, we hypothesized

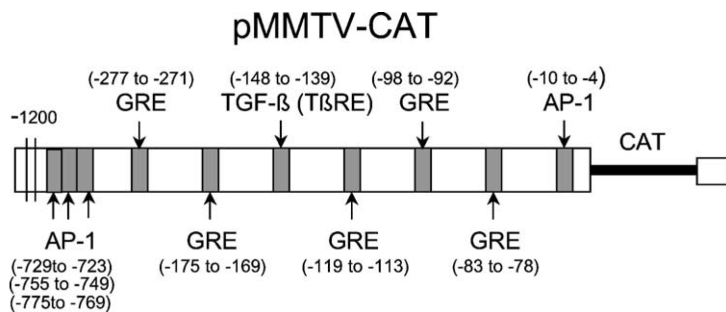

Fig. 6. Diagrammatic representation of the pMMTV-CAT Plasmid. The pMMTV-CAT promoter is a long (∼1200 bp) regulatory sequence. The location of the TGF-β-responsive element (TβRE), and the binding sites for GR and AP-1, are shown on the pMMTV-CAT promoter. The TβRE, and the binding sites for GR and AP-1 were obtained using computerized homology search by MacVector™ 6.5.3 software program.

that AP-1-mediated repression of GR at the *MMTV* promoter must occur though direct binding of AP-1 to its cognate response element. As an initial test of this hypothesis, we used curcumin to inhibit the expression of AP-1 in LMCAT cells. Huang et al. [19] demonstrated that curcumin, a potent inhibitor of tumor promotion, inhibits c-Jun expression, but not that of the c-Fos gene. Furthermore, curcumin inhibits the expression and post-translational modification of the c-Jun protein by inhibiting c-Jun N-terminal kinase [19,51]. However, later studies by Kakar and Roy [20] and Huang et al. [21] have shown that TPA-induced expression of c-Fos and c-Jun proteins was inhibited by curcumin in NIH 3T3 cells and in mouse skin, respectively. In the experiments of Fig. 7A, we treated LMCAT cells with various concentrations of curcumin (0.625–5 μM) for 3 h followed by Dex (100 nM) for 20 h and measurement of CAT activity. Curcumin up to 5.0 μM concentration showed no effect on basal and Dex-induced *CAT* gene expression (Fig. 7A). To determine the effect of curcumin on TGF-β inhibition of GR-mediated *CAT* gene expression, LMCAT cells were pretreated in the absence and presence of curcumin (1.0 μM) and TGF-β_1 (5 ng/ml) for 3 h, followed by incubation with Dex (100 nM). As shown in Fig. 7B, curcumin completely abolished the inhibitory effect of TGF-β on GR-mediated gene expression without affecting basal or GR activity in the absence of TGF-β. These findings clearly demonstrate the existence of mutual antagonistic cross-talk

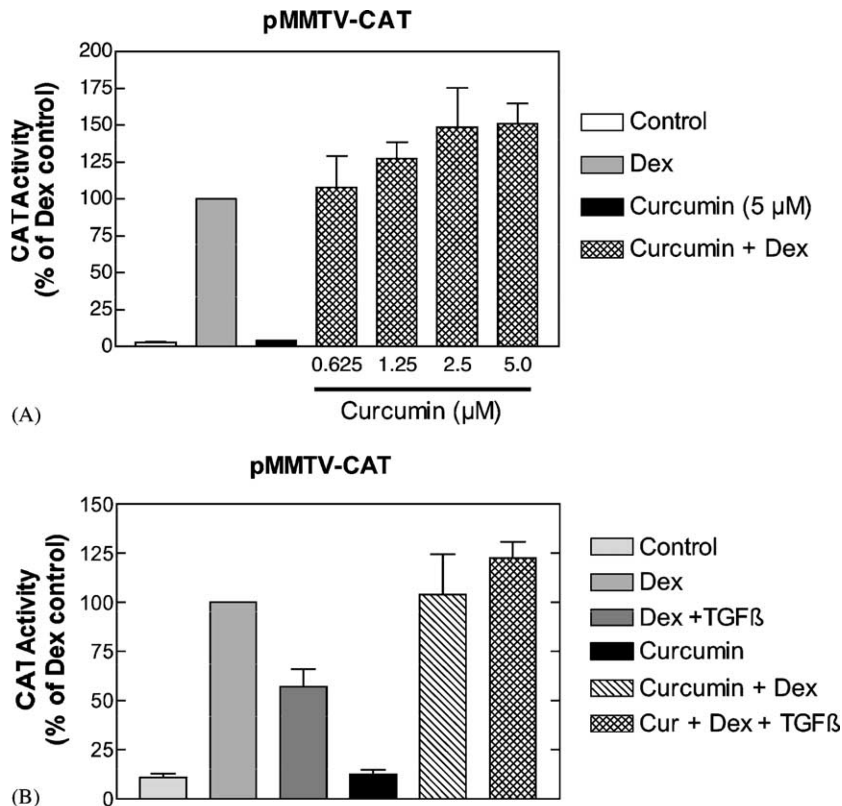

Fig. 7. Curcumin reverses TGF-β inhibition of GR-mediated *CAT* gene expression. (A) Curcumin has no effect on GR-induced *CAT* gene expression in the absence of TGF-β. LMCAT cells were treated with curcumin at the indicated concentrations for 3 h, followed by incubation with and without Dex (100 nM) for an additional 20 h. The results shown represent the mean ± S.E.M. of three independent experiments; (B) effects of curcumin on TGF-β inhibition of GR. LMCAT cells were exposed to various combinations of TGF-β_1 (5 ng/ml), curcumin (1.0 μM) and Dex (100 nM), as indicated, followed by assay for CAT. The data represent the mean ± S.E.M. of three independent experiments.

between GR and TGF-β by a mechanism involving AP-1 in these cells.

3.8. Curcumin inhibits TGF-β-induced AP-1 DNA-binding activity

Abrogation by curcumin of the inhibitory effect of TGF-β on GR-mediated *CAT* gene expression (Fig. 7B) suggested that TGF-β might increase the expression and binding activity of AP-1 (c-Jun/c-Fos). To test this, we performed gel shift assays using a synthetic AP-1 oligonucleotide. As shown in Fig. 8, marked binding of nuclear proteins to the AP-1 oligonucleotide was observed in TGF-β-treated cells, and this binding was inhibited by curcumin. On the other hand, nuclear extracts from control and curcumin-treated cells showed only a minimal, basal binding to the AP-1 sequence. To further confirm that the protein-DNA complex contains AP-1, gel shift assays were carried out in the presence of c-Jun and c-Fos antibodies. Treatment of the extracts with these antibodies inhibited the binding of AP-1 to the AP-1 oligonucleotide in the TGF-β-treated cells, indicating that both c-Jun and c-Fos are present in the AP-1 complex (Fig. 8).

It has been shown that TGF-β inhibition of the transin, c-myc, and collagenase genes is mediated by the induction of c-Fos and subsequent binding of c-Fos to TβRE sequences [52]. As the *MMTV* promoter contains a consensus TβRE sequence (Fig. 6), we tested binding of AP-1 to the TβRE sequence in TGF-β-treated cells (Fig. 9). As expected, nuclear extracts from TGF-β-treated cells showed increased binding of AP-1 to AP-1 oligonucleotide (ARE). In

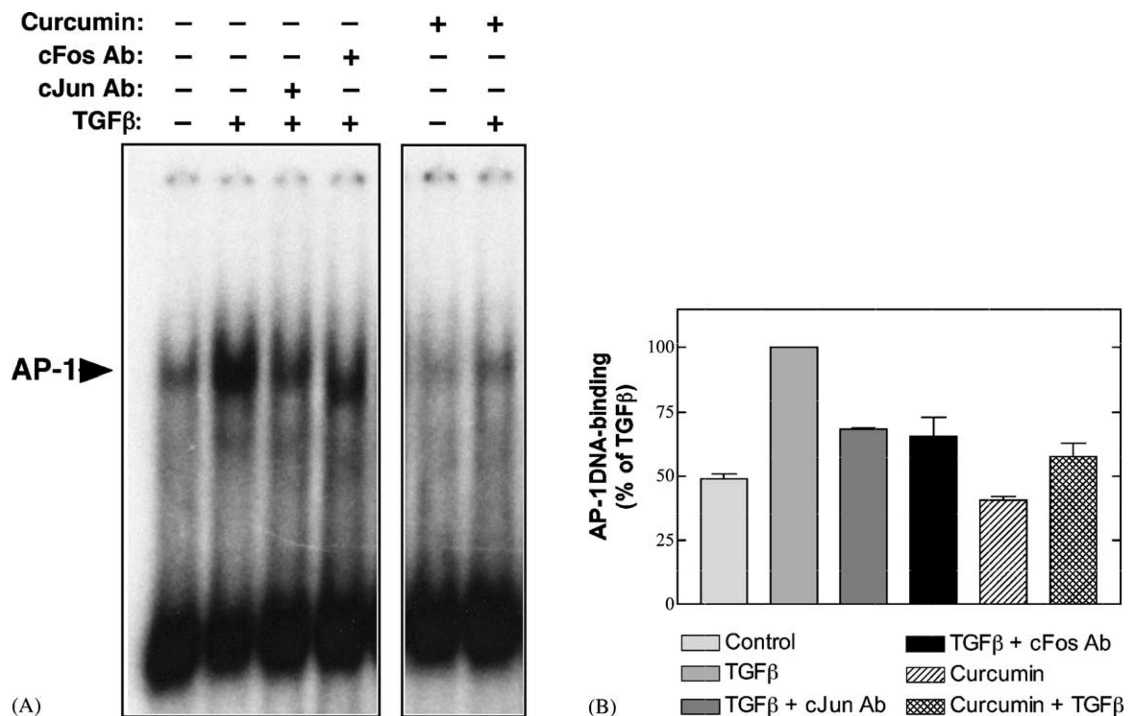

Fig. 8. Curcumin and antibodies to c-Jun and c-Fos inhibit TGF-β-induced AP-1 binding to DNA. LMCAT cells were treated with and without curcumin (1.0 μM) in the absence or presence of TGF-β_1 (5 ng/ml). After 3 h, nuclear extracts were prepared and EMSA performed with ^{32}P-labeled oligonucleotides containing the AP-1 sequence. Replicate samples were incubated with antibodies to c-Jun and c-Fos prior to EMSA. (A) Autoradiogram of typical results. Samples 5 and 6 of this experiment were run on a separate portion of the same gel and all samples were exposed to the same film. Arrow indicates band corresponding to AP-1-bound DNA; (B) quantitation of results by densitometric scanning of the autoradiograms and normalization to TGF-β alone. The results shown represent the means ± S.E.M. of two independent experiments.

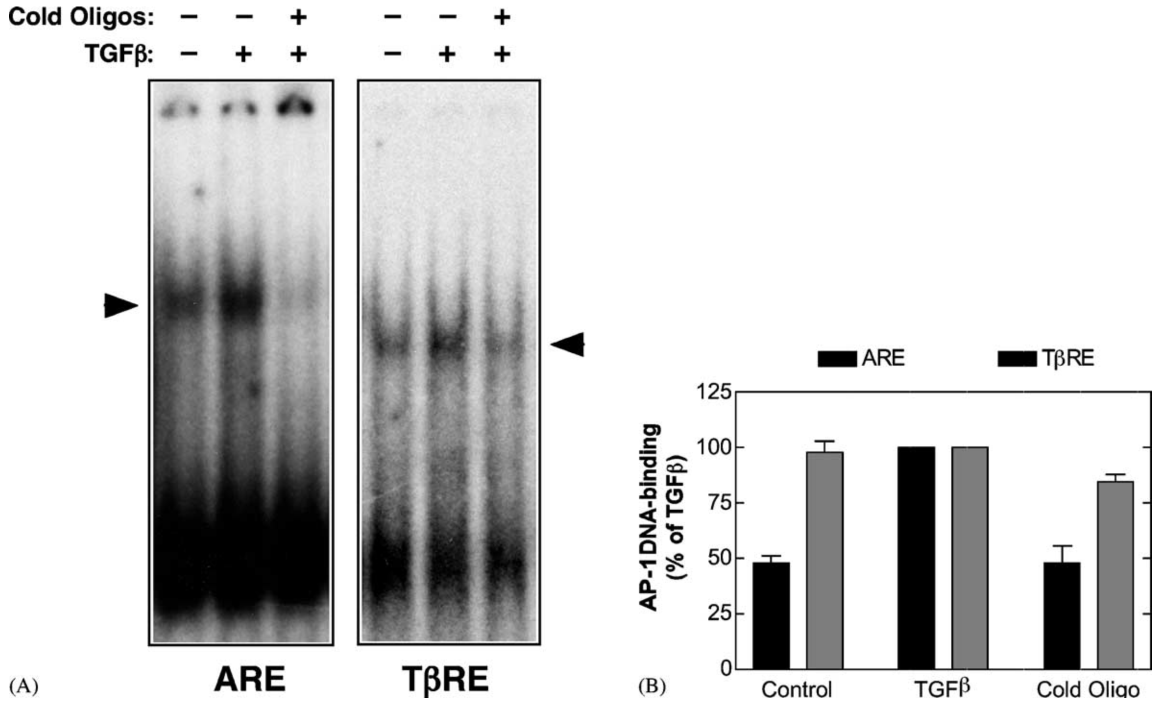

Fig. 9. Comparison of TGF-β-induced AP-1 binding to AP-1 and TβRE oligonucleotide sequences. LMCAT cells were treated with or without TGF-β_1 (5 ng/ml) for 3 h, followed by EMSA with ^{32}P-labeled oligonucleotides corresponding to consensus AP-1 (ARE) and TβRE sequences. Unlabeled "cold" AP-1 and TβRE oligonucleotides were used as competitors. (A) autoradiogram of typical results. Arrows indicate oligonucleotide-bound complexes; (B) quantitation of results by densitometric scanning of the autoradiograms. Results shown represent the mean ± S.E.M. of three independent experiments.

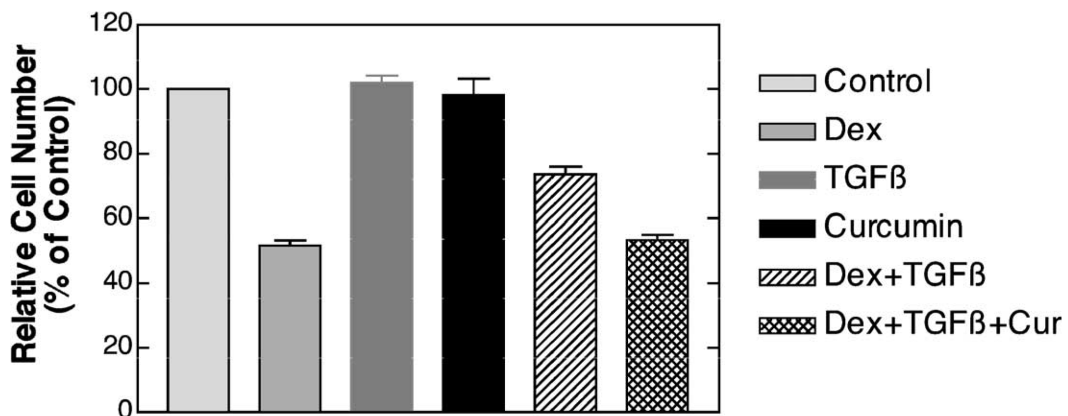

Fig. 10. Curcumin abrogates TGF-β inhibition of Dex-induced growth suppression in L929 cells. L929 cells (5×10^3 cells/well) were incubated for 7 days in the presence of Dex (100 nM), TGF-β_1 (5 ng/ml), curcumin (1 μM) and the indicated combinations of each, followed by MTT colorimetric assay. The data is representative of two independent experiments. In each experiment, cell densities in three replicate wells were measured per condition. Thus, each value corresponds to the mean ± S.E.M. of six wells, with 100% growth representing untreated control cells.

contrast, no measurable increase was observed in the binding of TβRE oligonucleotide to nuclear proteins from TGF-β-treated cells, suggesting that the TβRE-sequence is not involved in the mechanism by which TGF-β inhibits GR-mediated functions in these cells.

3.9. Curcumin abrogates TGF-β inhibition of Dex-induced growth suppression

To determine whether curcumin can abrogate TGF-β inhibition of Dex-induced growth suppression, L929 cells were treated with and without curcumin in the presence and absence of TGF-β and Dex. Fig. 10 shows that curcumin abolished TGF-β inhibition of Dex-induced growth suppression. On the other hand, curcumin showed no effect on control growth or Dex-induced growth suppression. Taken as a whole, these data suggest that inhibition of GR through TGF-β-induced AP-1 activity is indeed the mechanism by which TGF-β inhibition of Dex-induced growth suppression involves activation of AP-1.

4. Discussion

Using L929 murine fibrosarcoma cells, we have demonstrated that TGF-β can antagonize hormone-induced GR transcriptional activity at the *MMTV* promoter through a mechanism involving activation of the AP-1 transcription factor. We base this conclusion on several salient observations. First, TGF-β inhibition of GR activity at the *MMTV* promoter was observed after hormonal activation and presumptive nuclear translocation of the receptor, suggesting that a late functional stage, such as transactivation, was being affected. Second, TGF-β inhibition of GR was not observed with the minimal pGRE$_2$E1B promoter. Thus, it is likely that DNA-binding transcription factors other than GR are required for this effect. Third, the *MMTV* promoter was found to contain several consensus AP-1 response elements, and activation of AP-1 DNA-binding activity was observed in response to TGF-β treatment of cells. Lastly, curcumin, a potent inhibitor of AP-1, completely abrogated both TGF-β activation of AP-1 and the inhibitory effect of TGF-β on GR-induced *MMTV* promoter activity.

At a minimum, the above findings would indicate that inhibition of GR by TGF-β can occur through this mechanism in our L929 cells, at least with respect to the *MMTV* promoter. Yet a broader implication would be that such a mechanism may also be operating at promoters of one or more endogenous genes, whose coordinated regulation may be responsible for important physiological responses to these signals. Although in this work we do not provide evidence of such genes, we have found evidence that control of cellular proliferation in L929 cells is controlled by this mechanism, as glucocorticoid repression of growth was antagonized by TGF-β and curcumin reversed the growth factor effect. Thus, this mechanism appears to regulate an important function in L929 cells and may, therefore, regulate additional physiological responses in these cells and others.

Our results suggest that activated AP-1 can repress GR transcriptional activity—a result that is consistent with some reports, but not others. For example, Vitamin D receptor activity is enhanced by AP-1 in osteoblasts [31], while overexpression of Fos and Jun caused inhibition of GR-dependent reporter gene activity [53]. In NIH-3T3 fibroblast cells, activation of AP-1 caused a similar inhibition of GR at the *MMTV* promoter [54]. Yet, in studies using the same *MMTV* promoter in T cell lines, Dex-induced transcription was actually augmented by AP-1 [54]. Therefore, it appears that many factors other than simple activation of GR and AP-1 are responsible for the net effect of their interaction. Although these factors are far from resolved, it has recently been shown by Pearce et al. [55] that the cross-talk between GR and AP-1 depends upon the relative position of GR and AP-1 binding sites within a promoter. When the two sites are both present, but not closely juxtaposed (from 26 to 210 bp), they act synergistically regardless of the composition of AP-1 (c-Jun/c-Jun; c-Jun/c-Fos). However, when binding sites for GR and AP-1 are closely juxtaposed (separated by 14–18 bp), they behave as composite GRE, i.e. GR is synergized with c-Jun/c-Jun and repressed with c-Jun/c-Fos. These findings suggest that AP-1 plays a bifunctional role on GR-dependent transcriptional activation, causing either synergistic or antagonistic activity depending on the context of promoter binding site. Though the Pearce et al. model may be intriguing, it does not adequately explain our results, as the GR and AP-1

binding sites in *MMTV* promoter are not closely juxtaposed (Fig. 5), yet AP-1 repression of GR still occurs.

If AP-1 is repressing GR activity in our system, how does this happen? Many models have been proposed to explain the negative interactions between AP-1 and GR at various promoters and in different cell types. They are: (1) direct protein–protein interaction causing mutual inhibition of DNA binding; (2) tethering of AP-1 to DNA-bound GR; (3) competition for common co-activators; and (4) co-occupancy by AP-1 and GR at adjacent or overlapping recognition sites. With respect to our results, it appears that mutual inhibition of DNA binding or tethering of AP-1 to GR are not plausible explanations, as TGF-β did not inhibit GR-mediated *CAT* gene expression in cells stably transfected with $pGRE_2E1B$-CAT minimal promoter. The lack of repression with this promoter also suggests that competition for common co-activators is not likely, assuming that activated AP-1 not localized to the region of chromatin containing $pGRE_2E1B$-CAT would have equal access to co-activators. Since the *MMTV* promoter does not contain composite GRE, it is also unlikely that both GR and AP-1 are competing for overlapping GRE and AP-1 binding sites (composite GRE) in the *MMTV* promoter. For these reasons, we believe the most plausible mechanism of repression involves co-occupancy of GR and AP-1 within the *MMTV* promoter—a mechanism that will be further tested by functional mapping of the putative AP-1 binding sites within the *MMTV* promoter (Fig. 6).

In most cell contexts, GCs exert anti-proliferative effects, which has prompted their use clinically as part of anticancer therapy for several types of tumors. However, the molecular basis of the anti-proliferative actions of these steroids is not fully understood. Since GR can both enhance and repress gene expression, two models have been proposed. One holds that the hormone-activated GR induces anti-mitogenic factors, such as the cyclin-dependent kinase inhibitors p27kip1 and $p21^{cip1}$, which cause cell cycle arrest [45,56]. An alternative model is that GR can cause growth arrest, not as a transcriptional activator, but as a transcriptional repressor—interfering with the expression of mitogenic factors, such as cyclins and kinases [56]. TGF-β, on the other hand, has been shown to have both growth-stimulatory and growth-inhibitory properties. For example, in keratinocytes, TGF-β down-regulates transcription of c-myc oncogene [57] and the cyclin A and the B-myb genes [58], while up-regulating the cyclin-dependent kinase inhibitors $p15^{ink4B}$ and $p21^{cip1}$ [59]. These effects are consistent with the potent anti-proliferative actions of TGF-β on other epithelial cells. In contrast, TGF-β induces expression of the growth promoting, immediate-early genes c-Jun and c-Fos in NIH3T3 and AKR-2B fibroblastic cells, whose proliferation is induced by the growth factor [60]. Thus, the effects of TGF-β seen in L929 cells appear to be typical of the effects seen in fibroblastic cells, except for the fact that TGF-β alone did not promote proliferation in our cells (Fig. 9A).

In summary, our results suggest that cross-talk between GR and TGF-β can be explained, at least in part, by modulation of AP-1 activity. Increased activity of AP-1 by TGF-β prevented GR-mediated gene expression, as well as the inhibitory effect of GCs on cell proliferation. As TGF-β, in particular, can have highly-divergent effects on cells, depending on their tissue lineage and other, as yet, unknown factors, our study may help to foster a better understanding of the tissue-specific differences that exist with respect to GR and TGF-β cross-talk mechanisms. Such understanding may facilitate the eventual development of new drugs and regimens designed to selectively target tissue and organ systems.

Acknowledgements

Supported by grants to Edwin R. Sánchez by the National Institutes of Health (DK43867) and the National Science Foundation (MCB-9905117).

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