Biochemical Pharmacology, Vol. 60, pp. 1677–1684, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00507-4

Differential Regulation of a Fibroblast Growth Factor-Binding Protein by Receptor-Selective Analogs of Retinoic Acid

*Bryan J. Boyle, Violaine K. Harris, Emmanuelle D. E. Liaudet-Coopman, Anna T. Riegel and Anton Wellstein**

Departments of Oncology and Pharmacology, Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC 20007, U.S.A.

ABSTRACT. We have demonstrated earlier that a secreted fibroblast growth factor-binding protein (FGF-BP) can enhance angiogenesis and promote tumor growth *in vivo*. Furthermore, we found that FGF-BP expression in squamous cell carcinoma (SCC) is reduced by concentrations of retinoids that are effective in the treatment of SCC and that this repression can occur at the transcriptional and post-transcriptional level. To further examine the mechanism of regulation of FGF-BP by retinoids and the role played by retinoid receptor subtypes, we utilized retinoic acid receptor (RAR)-selective (TTNPB) and retinoid X receptor (RXR)-selective (LG100268) ligands. In ME-180 SCC cells, FGF-BP mRNA was down-regulated by TTNPB with an IC_{50} value of 1 nM, whereas transcription was only repressed at 10,000-fold higher concentrations ($IC_{50} > 10$ μM). This suggests that the major effects of retinoids on FGF-BP occur at the post-transcriptional level. In four additional SCC cell lines, FGF-BP was also down-regulated by TTNPB with IC_{50} values of ≤ 1 nM, demonstrating that RAR receptors can modulate FGF-BP mRNA levels very effectively in SCC cells. The RXR-selective ligand on its own was only effective in two of the five cell lines (IC_{50} of ≈ 1 nM). In all of the SCC cell lines, a low concentration of RAR sensitized FGF-BP mRNA to treatment with the RXR ligand and the combination of the RXR and RAR ligands enhanced the efficacy beyond that of the individual ligands. We conclude that RAR receptors are major regulators of FGF-BP mRNA at the post-transcriptional level and propose that an RAR-induced gene product mediates the RXR effects on FGF-BP mRNA. BIOCHEM PHARMACOL **60**;11:1677–1684, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. FGF-BP; FGF; retinoids; RXR; RAR; angiogenesis

Retinoids are a family of compounds containing both natural and synthetic analogs of vitamin A. They have been shown to regulate epithelial growth and differentiation as well as inhibit progression of neoplasms including carcinoma of the squamous epithelium [1–3]. Retinoids have also been shown to prevent carcinogenesis in several epithelial tissues, including lung, trachea, skin, and the oral mucosa [4, 5]. In addition, they have been used chemotherapeutically in several types of malignancies including SCC† [6–10].

Retinoid receptors act as ligand-activated *trans*-activating factors, mediating their effects by control of gene expression. There are two classes of retinoid receptors, RARs and RXRs, and both are members of the steroid/thyroid hormone/vitamin D family of receptors. Each class of retinoid receptor contains at least three subtypes, RAR α, β and γ, and RXR α, β and γ (reviewed in Ref. 11). Recently, synthetic analogs of retinoids have been described that selectively activate each class of retinoid receptor [12–15]. Two of these compounds are TTNPB and LG100268, which, respectively, activate the RAR and RXR classes of receptors [13, 16], and these analogs have proven to be useful in the molecular dissection of the retinoid pathway in control of gene expression.

The mechanisms through which retinoids act to inhibit SCC growth and development are not understood clearly. Recent evidence [17] suggests that in ME-180 cervical SCC, inhibition of angiogenesis is an important factor in prevention of tumor growth in nude mice. As polypeptide growth factors have been shown to play a major role in angiogenesis, they are likely targets for the actions of retinoids. However, retinoids have not been shown to significantly down-regulate the expression of classical growth factors like FGFs. An alternative target for retinoids could be helper proteins, which facilitate the actions of

* Corresponding author: Anton Wellstein, M.D., Ph.D, Department of Oncology, Research Bldg. E315, Lombardi Cancer Center, Georgetown University, 3970 Reservoir Road, Washington, DC 20007-2197. Tel. (202) 687-3672; FAX (202) 687-4821; E-mail: wellstea@georgetown.edu

† Abbreviations: ATRA, all-*trans*-retinoic acid; FGF, fibroblast growth factor; FGF-BP, fibroblast growth factor-binding protein; IMEM, improved minimal essential medium; LG100268, 6-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl]nicotinic acid; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RXR, retinoid X receptor; SCC, squamous cell carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; and TTNPB, (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid.

Received 13 March 2000; accepted 5 June 2000.

polypeptide growth factors. One such protein is a secreted binding protein for FGFs (FGF-BP), which has been shown to bind FGF1 and FGF2 in a non-covalent reversible manner [18], and modulates their activity. Expression of FGF-BP in a non-tumorigenic cell line (SW-13) has been shown to increase soft-agar colony formation in these cells, as well as confer tumorigenic ability when injected into nude mice [19]. Conversely, selective reduction of FGF-BP in ME-180 SCC cells with ribozymes retards tumor growth *in vivo* [20]. In addition, expression of FGF-BP in these cells has been shown to solubilize endogenous bFGF from extracellular storage, allowing it to reach its receptor [21].

FGF-BP mRNA has been shown to be expressed at high levels in both SCC tissues and cell lines [21], which makes SCC cells an ideal model for the study of retinoid regulation of FGF-BP mRNA. We have shown that administration of the RAR/RXR pan-activator, ATRA, can down-regulate FGF-BP mRNA in both a concentration- and time-dependent manner [22].

Our current work shows that FGF-BP mRNA down-regulation at the transcriptional levels requires greater than micromolar levels of RAR or RXR agonists. In contrast, the post-transcriptional degradation of FGF-BP mRNA can be induced by nanomolar concentrations of TTNPB in several SCC lines. The concentrations of TTNPB required for this effect are on the same order of magnitude as those required for induction of a promoter harboring an RARE. This suggests that a retinoid induced cellular factor is responsible for the degradation of FGF-BP. Interestingly, administration of LG100268 (RXR-selective) ligand causes FGF-BP mRNA down-regulation in only two of the five cell lines tested. However, in all of the SCC lines, the combination of TTNPB and LG100268 gave the largest repression of FGF-BP mRNA levels. Overall our data indicate that post-transcriptional degradation of FGF-BP mRNA will be the predominant mechanism for reducing this angiogenic factor during therapy *in vivo*. Such down-regulation could be achieved in most SCC using submicromolar concentrations of RXR specific ligands in combination with low concentrations of sensitizing amounts of RAR agonist. This may have relevance to retinoid chemotherapy of SCC as RXR-selective ligands have significantly less side-effects in humans than RAR-selective ligands.

MATERIALS AND METHODS

Cell Lines

The squamous cell carcinoma cell lines ME-180, A431, FaDu, and SCC-25 were obtained from the American Type Culture Collection. The SqCC/Y1 squamous cell carcinoma cell line was a gift from Dr. Reuben Lotan, MD Anderson Cancer Center. Cells were grown in IMEM (Biofluids, Inc.) with 10% fetal bovine serum (Life Technologies, Inc.), with the exception of SqCC/Y1 cells, which were grown in Dulbecco's minimal essential/F12 medium (DMEM/F12) in 10% fetal bovine serum.

Compounds

ATRA, LG100268, and LG100815 were the gifts of Ligand Pharmaceuticals, Inc. TTNPB was acquired from Biomol Research Laboratories, Inc.

Transient Transfection of ME-180 Cells

Twenty-four hours before transfection, ME-180 cells were plated in 6-well plates in IMEM, 10% fetal bovine serum at a density of 750,000 cells/well. For each transfection, 1.0 μg FGF-BP (−118/+62) promoter/luciferase construct [23] or ΔMMTV-TRE Luc [15, 24] and 8 μL of Lipofectamine reagent (Life Technologies, Inc.) were combined in 200 μL of IMEM, and liposome–DNA complexes were allowed to form at room temperature for 30 min. Volume was increased to 1 mL with IMEM, the mixture was added to the rinsed cells, and the cells were incubated for 3 hr at 37°. Cells were washed and incubated in IMEM for 3 hr and then treated for 18 hr with Me_2SO alone or in the presence of TPA and/or retinoids at concentrations indicated in the figure legends. Transfection efficiency was determined by co-transfection with 0.2 ng of a CMV Renilla luciferase vector reporter vector (Promega). Cells were lysed by scraping into 150 μL of passive lysis buffer (Promega), and cell debris was removed by brief centrifugation. Twenty microliters of extract was assayed for both firefly and Renilla luciferase activity using the Dual Luciferase reporter assay system™ (Promega). Light intensity was measured in a Monolight 2010 luminometer. Protein concentration of cell extracts was determined by the Bradford assay.

RNA Extraction

ME-180 cells were grown to 80% confluence in 75-cm^2 tissue culture flasks, washed twice in serum-free IMEM, and then treated with retinoids in Me_2SO or vehicle alone for 24 hr in serum-free medium. Total RNA was isolated by lysing the cells with RNA Stat-60 (Tel-Test 'B', Inc.), which contains guanidinium thiocyanate and phenol in a monophase solution. Homogenates were treated with chloroform, separating them into aqueous and organic phases, with total RNA present exclusively in the aqueous phase. Precipitation of RNA was carried out by the addition of isopropanol and washing with ethanol. Total RNA then was resuspended in water.

Northern Analysis of RNA

Thirty micrograms of total RNA was separated by electrophoresis in 1.2% formaldehyde-agarose gel and then blotted onto nylon membranes (Micron Separations, Inc.). The blots were prehybridized in 6X SSC (0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 5X Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA], 50% formamide, and 100 μg/mL of sonicated salmon sperm DNA (Life Technolo-

gies, Inc.) for 4 hr at 42°. Hybridization was carried out overnight at 42° in the same buffer. After hybridization, blots were washed three times with 2X SSC and 0.1% SDS for 10 min at 42° and once with 1X SSC and 0.1% SDS for 20 min at 65°. Autoradiography was performed with intensifying screens at −80°. Quantitation of signal was performed using phosphorimaging analysis (Molecular Dynamics), and normalized to GAPDH control gene. Blots were stripped by boiling two times for 10 min in 0.1X SSC and 0.1% SDS. Hybridization probes were made by random-primed DNA labeling (Amersham Life Sciences) of a purified, insert fragment from human FGF-BP [18] and human GAPDH (CLONTECH). The final concentration of the labeled probes was always greater than 2×10^{-6} cpm/mL of hybridization solution.

Nuclear Run-On Analysis

ME-180 cells were grown to 80% confluence and treated with TTNPB or LG100268 at the indicated concentrations for 1 hr; then nuclei were isolated, and nuclear run-on experiments were performed as described previously [22, 23]. Equal amounts of radioactivity (1×10^7 cpm) were hybridized to nitrocellulose filters containing 3 μg of FGF-BP plasmid or the actin control plasmid as described previously [22, 23]. After washing, the amount of radioactivity present in each slot was determined using a PhosphorImager after overnight exposure, and the autoradiograms were exposed for 1–3 days with intensifying screens.

RESULTS

Effects of RAR and RXR Specific Ligands on BP Gene Promoter Activity

We have determined previously with nuclear run-on analysis that the FGF-BP gene is induced by the phorbol ester TPA in ME-180 SCC [23]. The TPA induction is driven by a 118 bp promoter fragment upstream of the transcription initiation site [23]. Interestingly both the basal and TPA-induced levels of FGF-BP mRNA can be suppressed by ATRA with an IC_{50} of 2×10^{-7} M [22]. To determine if retinoid selective ligands were direct inhibitors of FGF-BP gene transcription and to determine the involvement of the retinoid receptor subtypes in the response, we tested the effects of TTNPB and LG100268 on the FGF-BP gene promoter. Using the −118 bp promoter fragment fused to a luciferase reporter gene, we determined that the basal (Fig. 1A) and TPA-induced (Fig. 1B) transcription from the FGF-BP promoter could only be suppressed by concentrations of 10^{-5} M ligand. The data with TTNPB at 10^{-5} M are not shown because this concentration is toxic to cells. Although up to 50% transcriptional repression was observed with the maximum tolerated concentration of RAR or RXR ligands, it is clear that this effect does not explain why 100-fold lower concentrations of retinoids can suppress FGF-BP mRNA.

One possible mechanism to explain the recalcitrance of the FGF-BP promoter response to retinoids could be a relatively low level of RAR or RXR receptors in these cells or the presence of inactive receptors. To test this possibility, we examined the response of a ΔMMTV-TRE promoter that harbors a palindromic RARE and that is induced by retinoids such as TTNPB and LG100268 [15, 24]. In contrast to the FGF-BP promoter, the ΔMMTV-TRE was stimulated markedly by either 10^{-7} M TTNPB or LG100268 (Fig. 1C). In fact, a combination of both ligands at this concentration was able to produce an approximately 15-fold induction similar to that achieved by 10^{-6} M ATRA. These results indicate that the insensitivity of the transcriptional repression of the FGF-BP promoter is not due to lack of functional RAR or RXR receptors in these cells.

It should be noted that the data with the transfected FGF-BP promoter reflect the regulation of the endogenous gene since only high concentrations (10^{-5} M of either ATRA [22] or LG100268 were able to directly suppress the FGF-BP gene transcription as measured in a run-on assay (Fig. 1D). In addition, also consistent with Fig. 1A, TTNPB (10^{-7} M) had no effect on the endogenous FGF-BP gene transcription (Fig. 1D).

Effects of TTNPB and LG100268 on FGF-BP mRNA Regulation in ME-180 Cells

In previous studies using actinomycin D blockade of transcription, we determined that FGF-BP mRNA has a half-life longer than 18 hr in ME-180 cells. However, in the presence of ATRA, the FGF-BP mRNA is degraded rapidly, with a half-life of under 6 hr [22]. This degradation can be prevented by cycloheximide, suggesting that the specific degradation of FGF-BP mRNA requires a synthesized gene product [22]. To determine the relative role of the RAR and RXR classes of receptors in this novel retinoid-induced, post-transcriptional degradation mechanism, ME-180 cells were treated with TTNPB and LG100268, and the FGF-BP mRNA levels were determined by northern analyses (Fig. 2). Administration of TTNPB in a concentration range of 10^{-10} to 10^{-6} M caused a concentration-dependent down-regulation of FGF-BP mRNA with an IC_{50} of approximately 0.5 nM and a maximal effect to 60% of control levels at the highest concentration. Activation of RXR receptors by LG100268 under the same concentration range, however, did not exhibit FGF-BP mRNA down-regulation in these cells, even at 10^{-5} M. Interestingly, at 10^{-5} M LG100268 we did observe some transcriptional repression of the endogenous gene (Fig. 1D), but within the 24 hr of the experiment in Fig. 2 we saw no reduction in FGF-BP mRNA. Most likely this is due to the long half-life of the FGF-BP mRNA in these cells, which has been reported previously [22].

It has been demonstrated previously [22] that administration of ATRA, which causes activation of both RAR and RXR, produced a greater effect in ME-180 cells than did TTNPB at the same concentration (10^{-6} M). In this study, ATRA was able to down-regulate FGF-BP mRNA to

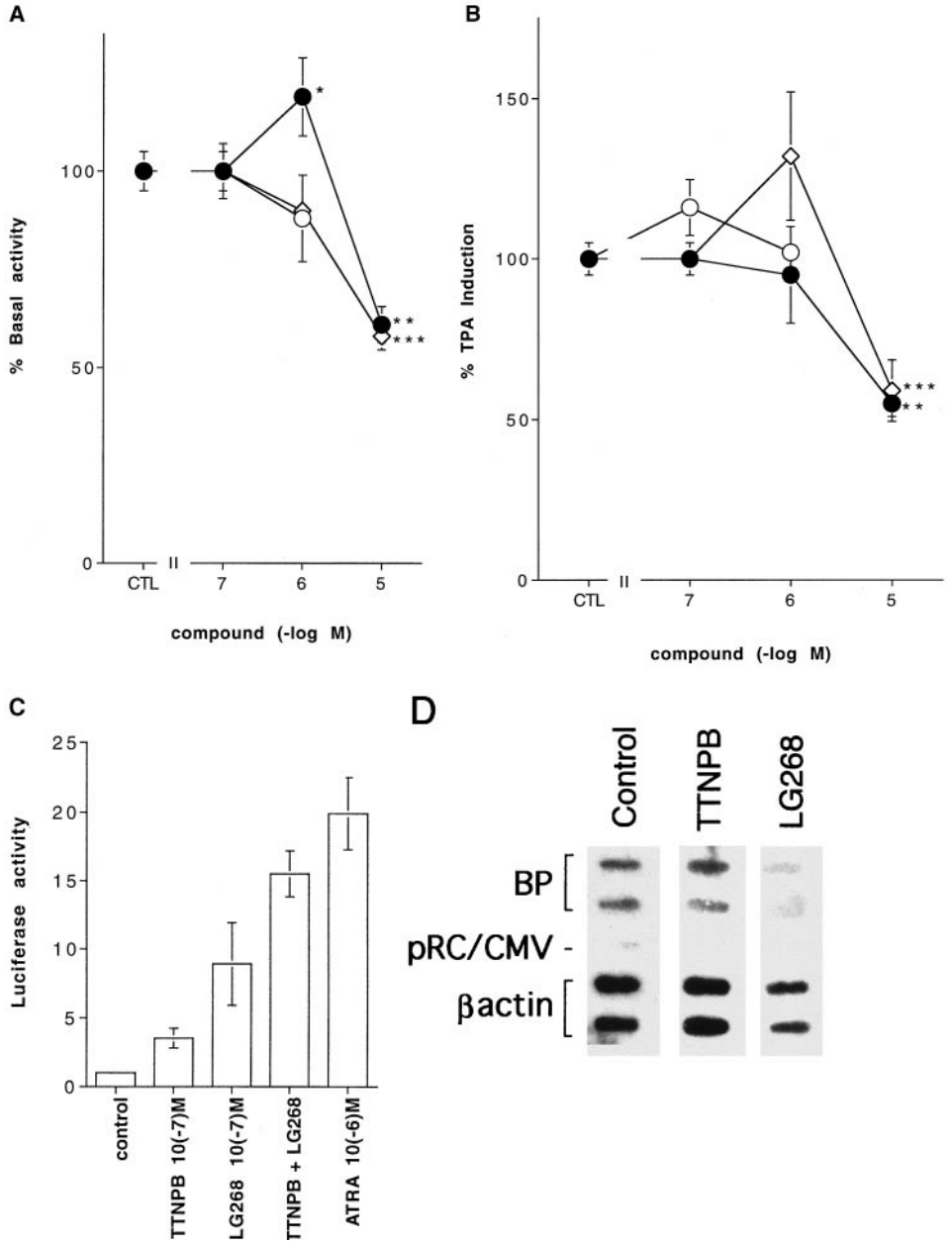

FIG. 1. Effects of retinoids on gene transcription in the ME-180 SCC line. ME-180 cells were transiently transfected with the −118/+62 FGF-BP promoter construct driving the luciferase reporter gene [23]. (A) Cells were treated with increasing concentrations of ATRA (filled circles), TTNPB (open circles) or LG100268 (open diamonds) for 6 hr. Cells were harvested, and luciferase activity was assessed (see Materials and Methods). (B) Cells were transiently transfected with the FGF-BP promoter construct with 10^{-7} M TPA in the absence or presence of increasing concentrations of ATRA, TTNPB, or LG100268 (symbols as above). In panels A and B, luciferase activity is expressed as percent of the basal or TPA-stimulated promoter activity, respectively. Control values (=100%) were 5000 (A) and 35,000 (B) light units/mg of protein, respectively. Means ± SEM from three separate experiments are shown. Key: (*), (**), and (***) represent a significant difference from control values at $P < 0.05$, 0.01, and 0.001, respectively (Student's *t*-test). (C) ME-180 cells were transiently transfected with the ΔMMTV-TRE-Luc plasmid, which contains a retinoid responsive palindromic thyroid hormone response element (TRE). Cells were treated with the retinoid ligands at the concentrations indicated for 6 hr and then harvested and assayed for luciferase activity. Results represent -fold induction (means ± SD) from a representative of two different experiments, each conducted in triplicate. The control value (=1-fold) was 7000 light units/mg of protein. (D) Effects of TTNPB and LG100268 on FGF-BP gene transcription as determined by nuclear run-on assays. Nuclei were isolated from ME-180 cells and treated with TTNPB (10^{-7} M) or LG100268 (10^{-5} M) for 1 hr. Nuclei then were isolated and analyzed as described in Materials and Methods. The experiment was performed twice. The levels of FGF-BP gene transcription (untreated control = 100%) corrected for actin loading were: TTNPB (10^{-7} M), 97%; and LG100268 (10^{-5} M), 45%.

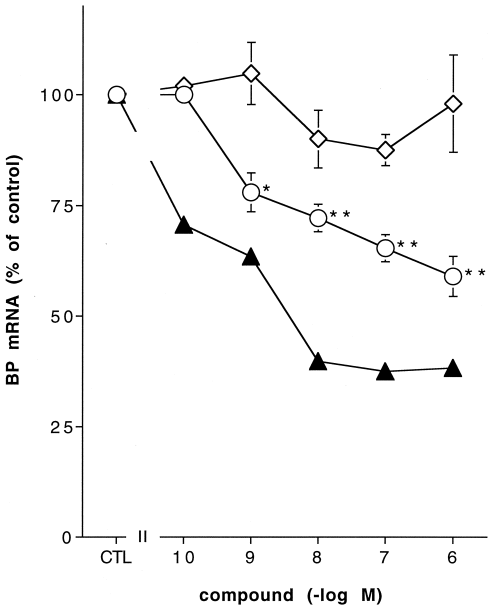

FIG. 2. Northern analysis of FGF-BP mRNA expression in ME-180 cells treated with RAR-selective TTNPB or RXR-selective LG100268. Cells were grown to 80% confluence and washed twice in serum-free IMEM. Then cells were treated for 24 hr with either vehicle, TTNPB (open circles), LG100268 alone (open diamonds) in a concentration range of 10^{-6} to 10^{-10} M, or with TTNPB in this concentration range in the presence of a constant concentration of LG100268 (10^{-7} M) (closed triangles). After treatment, cells were collected via trypsinization, and total RNA was isolated as described in Materials and Methods. Thirty micrograms of total RNA per sample was analyzed by northern analysis after transfer to a nylon membrane. The membrane was probed first with human FGF-BP cDNA, stripped, and probed with human GAPDH cDNA for use as a loading control. Signal intensities for both FGF-BP and GAPDH were quantified using a PhosphorImager (Molecular Dynamics, Inc.). The FGF-BP signal was adjusted according to GAPDH loading control intensities. Data from treated cells were compared with controls, which were set at 100%. The means ± SEM of five separate experiments for TTNPB and three separate experiments for LG100268 are shown. (* $P < 0.05$, ** $P < 0.01$; significantly different from control by ANOVA and the Tukey test.) A representative result from duplicate experiments is shown for the TTNPB plus LG100268 ligand combination.

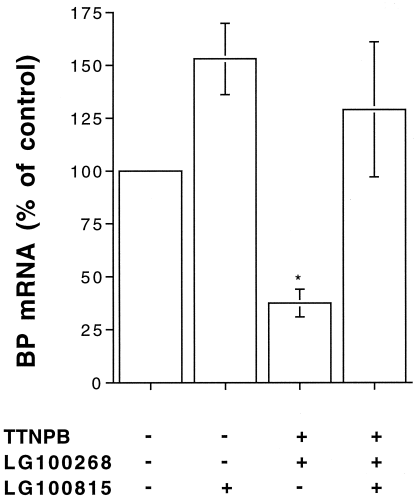

FIG. 3. Northern analysis of FGF-BP mRNA expression in ME-180 cells treated with the RAR antagonist LG100815 [25]. Cells were grown to 80% confluency and washed twice in serum-free IMEM. Then cells were treated for 24 hr with vehicle, LG100815 alone (10^{-6} M), the combination of TTNPB plus LG100268 (10^{-8} M/10^{-8} M), or this combination plus LG100815 (10^{-6} M). The effects of LG100268 and TTNPB alone in ME-180 cells are also shown in Fig. 2. After treatment, cells were collected via trypsinization, and total RNA was isolated as described in Materials and Methods. Thirty micrograms of total RNA per sample was analyzed by northern analysis as described in the legend to Fig. 2. The membrane was first probed with FGF-BP cDNA and then reprobed with GAPDH. The FGF-BP signal was adjusted according to the GAPDH loading intensities. Data from treated cells were compared with controls, which were set at 100%. The means ± SEM of three separate experiments are shown. Key: (*) significantly different from control values at $P < 0.05$ (Student's *t*-test).

40% of control levels, while only 60% of control levels was reached with TTNPB administration. This implies that activation of RXR receptors may play a role in FGF-BP mRNA regulation in these cells. To examine this further, ME-180 cells were treated with TTNPB in a concentration range of 10^{-10} to 10^{-6} M plus a constant concentration of LG100268 (10^{-7} M). This combination reached a maximal effect of 40% of control levels (Fig. 2). Interestingly, the TTNPB/LG100268 combination treatment is more potent than ATRA [22], with both combination treatments having an IC_{50} of approximately 2 nM, while the IC_{50} for ATRA was 200 nM [22].

Because RXR activation alone does not cause FGF-BP mRNA down-regulation in ME-180 cells, it appears that the effects of LG100268 are dependent upon concurrent RAR activation by TTNPB. To test this observation further, the RAR antagonist LG100815 [25] was employed in studies on ME-180 cells. Cells were treated with 100-fold excess of LG100815 plus a combination of TTNPB at 10^{-8} M and LG100268 at 10^{-8} M. Figure 3 shows the quantitation of the northern blots from this treatment. Administration of 10^{-6} M LG100815 alone to ME-180 cells exhibited an up-regulation of FGF-BP mRNA, most likely due to antagonism of endogenous retinoid levels in the media. As shown above, combination treatment of TTNPB plus LG100268 exhibited down-regulation of FGF-BP mRNA. Administration of the LG100815 RAR antagonist, however, reversed this down-regulation. This indicates that the RXR activation component of FGF-BP mRNA down-regulation in these cells is dependent upon RAR activation.

Effects of TTNPB and LG100268 on FGF-BP mRNA in other SCC Cell Lines

It has been shown previously that FGF-BP mRNA is down-regulated in several other SCC cell lines by administration of ATRA [22]. Because of this, we examined the effect of selective RAR and RXR activation in several other SCC lines, including the A431, FaDu, SCC-25, and

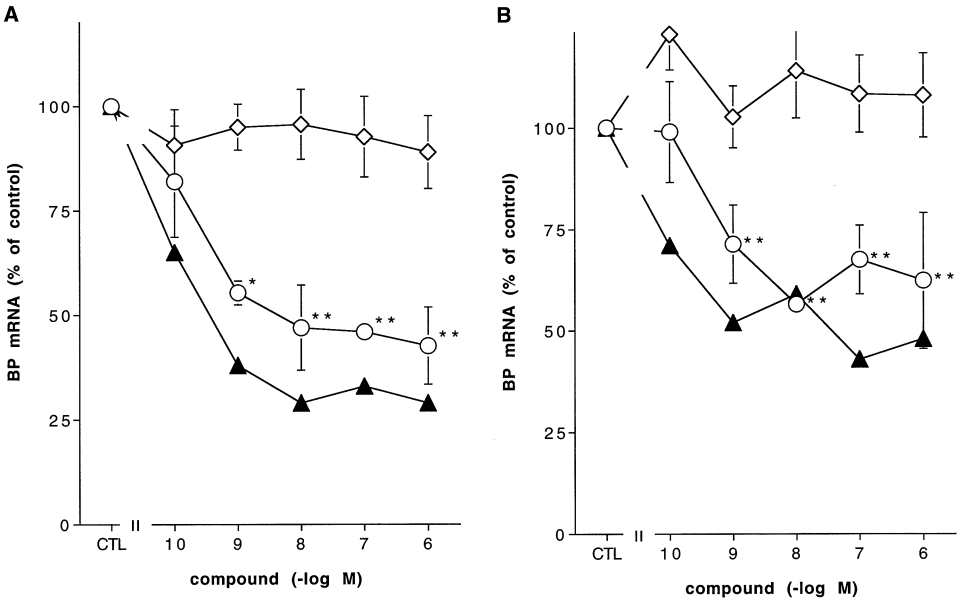

FIG. 4. Northern analysis of FGF-BP mRNA expression in (A) FaDu and (B) A431 cells treated with either RAR-selective TTNPB or RXR-selective LG100268. Cells were grown to 80% confluency and washed twice in serum-free IMEM. Then cells were treated for 24 hr with either vehicle, TTNPB, or LG100268 in a concentration range of 10^{-6} to 10^{-10} M. (Symbols are as described in the legend of Fig. 2.) After treatment, cells were collected via trypsinization, and total RNA was isolated as described in Materials and Methods. Thirty micrograms of total RNA per sample was analyzed by northern analysis as described in the legend to Fig. 2. The membrane was first probed with FGF-BP cDNA and then reprobed with GAPDH. The FGF-BP signal was adjusted according to the GAPDH loading intensities. Data from treated cells were compared with controls, which were set at 100%. The means ± SEM of three separate experiments for each compound and each cell line are shown. (* $P < 0.05$, ** $P < 0.01$; significantly different from control by ANOVA and the Tukey test.) A representative result from duplicate experiments is shown for the TTNPB plus LG100268 combination.

SqCC/Y1 cell lines. Panels A and B of Fig. 4 show quantitation of the northern analysis for concentration–response regimens of 10^{-10} to 10^{-6} M of either TTNPB or LG100268 administered to A431 or FaDu cells for 24 hr. This quantitation revealed that the differential regulation exhibited by RAR and RXR activation in ME-180 cells was also seen in A431 and FaDu cells. That is, FGF-BP mRNA was down-regulated by TTNPB, but not by LG100268. Similar to ME-180 cells, the combination caused a larger overall decrease in FGF-BP mRNA compared with TTNPB alone.

In contrast to the results in ME-180, FaDu, and A431 cells, the analysis of retinoid regulation in SSC-25 and SqCC/Y1 demonstrated that the lack of effect of LG100268 alone is not universal for all SCC lines. In fact, in both lines LG100268 alone was equipotent with TTNPB for the down-regulation of FGF-BP mRNA (Fig. 5, A and B). However, the combination of both ligands still caused a larger decrease in FGF-BP mRNA than either ligand alone (Fig. 5, A and B). Overall, our data indicate that RAR agonists alone can induce the post-transcriptional degradation of FGF-BP mRNA effectively. However, co-treatment with an RXR agonist can reduce the concentration of RAR ligand required to achieve maximum degradation (Table 1).

DISCUSSION

In the present study, we investigated the relative roles of the retinoid receptor subtypes in the transcriptional and post-transcriptional repression of FGF-BP gene expression. Our data indicate that both RXR and RAR ligands can only suppress the transcription of this gene at above micromolar concentrations. These levels of ligand would be hard to achieve clinically without incurring substantial side-effects. In contrast, transcriptional induction through a promoter harboring an RARE as well as the post-transcriptional degradation of FGF-BP mRNA occurs at retinoid concentrations several logs lower than those required for FGF-BP promoter repression. The difference in the retinoid concentrations required for FGF-BP transcriptional repression and FGF-BP mRNA degradation suggests that these processes occur by fundamentally different mechanisms and is consistent with current models of how retinoids repress and induce gene expression [11]. Transcriptional repression by retinoids is thought to occur through competition for a limiting cellular cofactor such as a transcriptional coactivator [26]. In contrast, transcriptional induction of gene expression can occur by direct effects of the ligand–receptor complex at its DNA binding site [11]. The concordance of the concentrations of ATRA and TTNPB required to induce ΔMMTV-TRE and the degradation of FGF-BP mRNA suggest that a gene product is induced by retinoids, which can then specifically degrade FGF-BP mRNA. This is consistent with the fact that the retinoid induction of degradation is cycloheximide sensitive [22]. However, if this latter model is correct, one would expect the degradation of FGF-BP mRNA to be induced by LG100268 since

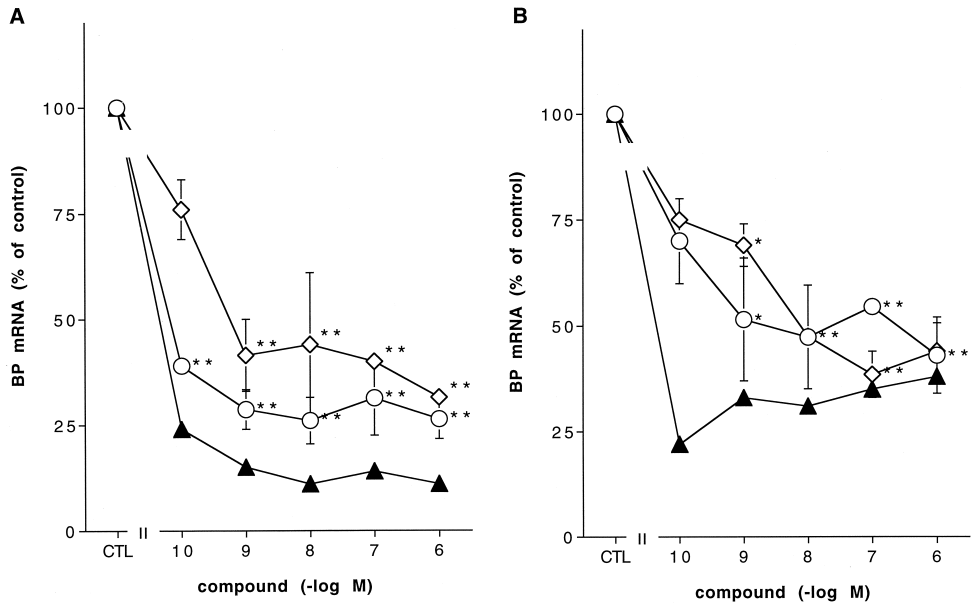

FIG. 5. Northern analysis of FGF-BP mRNA expression in (A) SCC-25 and (B) SqCC/Y1 cells treated with either RAR-selective TTNPB or RXR-selective LG100268. Cells were grown to 80% confluency and washed twice in serum-free IMEM. Then cells were treated for 24 hr with either vehicle, TTNPB, or LG100268 in a concentration range of 10^{-6} to 10^{-10} M. (Symbols are as described in the legend of Fig. 2.) After treatment, cells were collected via trypsinization, and total RNA was isolated as described in Materials and Methods. Thirty micrograms of total RNA per sample was transferred to nylon membrane and analyzed by northern analysis as described in the legend to Fig. 2. The membrane was first probed with FGF-BP cDNA and then reprobed with GAPDH. The FGF-BP signal was adjusted according to the GAPDH loading intensities. Data from treated cells were compared with controls, which were set at 100%. The means ± SEM of three separate experiments for each compound and each cell line are shown. (* $P < 0.05$, ** $P < 0.01$; significantly different from control by analysis of variance and the Tukey test.) A representative result from duplicate experiments is shown for the TTNPB plus LG100268 combination.

the palindromic ΔMMTV-TRE is fully responsive to this ligand in ME-180 cells.

Interestingly, despite the lack of effect of the RXR ligand alone in ME-180, FaDu, and A431 cell lines, the combination of TTNPB and LG100268 always produced the lowest levels of FGF-BP mRNA in all cell lines tested. The synergistic, but not additive effect of the RXR compound with RAR ligand can be explained if one invokes a model whereby a gene involved in the degradation of FGF-BP harbors an RARE that can be induced by an RAR:RXR heterodimer. Recently, it has been demonstrated that while this heterodimer cannot be activated by RXR ligand alone due to steric hindrance of the RXR ligand binding site, this can be relieved by binding of an RAR ligand that reveals the RXR binding site and allows the RXR ligand to bind [27]. Thus, combination of the two ligands exhibits a synergistic effect. However, if this is the mechanism of synergistic degradation of the FGF-BP mRNA, the question remains why the RXR ligand treatment alone has an effect in the SqCC/Y1 and SCC-25 lines?

TABLE 1. Comparison of the IC_{50} values for represssion of FGF-BP mRNA by receptor selective analogs of retinoic acid in various SCC cell lines

	TTNPB (T)	LG100268 (L)	T + L
Cell line		IC_{50} (nM)	
ME-180	1	> 1000	0.1
A431	6	> 1000	0.1
FaDu	6	> 1000	0.1
SCC-25	0.06	6	0.04
SqCC/Y1	0.1	1	0.06

The IC_{50} values for TTNPB, LG100268, or a combination of both drugs were calculated from the data presented in Figs. 2–5. The IC_{50} is defined as the concentration of a drug required to produce 50% of the maximal suppressive effect of that compound.

One possible explanation may be that RXR sensitive cell lines have relatively high levels of RAR that are sensitive to low levels of retinoid in the serum. This low level of ligand-bound RAR may be sufficient to reveal an effect of LG100268 when added to the media. However, our analysis of RAR α mRNA levels (data not shown) as well as those of other groups [28] has not demonstrated major differences in RAR levels in the SCC cell lines used in this study. Also, the addition of an RAR antagonist to the ME-180 cells caused an approximately 1.6-fold increase in FGF-BP mRNA, indicating substantial repression via the RAR by retinoids present in the serum. Despite this, the LG100268 was ineffective until TTNPB was added simultaneously.

Conversely, a relatively low level of active RXR molecules in the ME-180, FaDu, and A431 cells also does not seem a likely explanation of the difference in LG100268 effects, since the ΔMMTV-TRE construct is responsive to LG100268 alone in the ME-180 cells. In addition, our analysis of RXR α, β, and γ mRNA levels has revealed no major differences in these cell lines (data not shown). If the levels of RAR or RXR do not explain the differences in

susceptibility to LG100268, it may be possible that the RXR, heterodimerized with another nuclear receptor or orphan receptor, is able to induce the degradation of the FGF-BP at some point in the degradation pathway. RXR heterodimerizes with a number of known nuclear and orphan receptors [29] that could be involved. In this model the heterodimerization partner would be constitutively present in the SqCC/Y1 and SCC-25 lines, but this dimerization partner would be induced by RAR ligands in the ME-180, A431, and FaDu cell lines.

Whatever the mechanism of the different levels of the effectiveness of LG100268 in the various SCC lines, it is clear that the combination of a relatively low concentration of RAR ligand in combination with an RXR agonist can bring about the maximal repression of the angiogenic FGF-BP gene. The ability to use a small sensitizing dose of RAR ligand in combination with larger doses of RXR agonist is of therapeutic importance since the side-effects of retinoid therapy are more associated with the RAR ligands than with the RXR agonists.

These studies were funded, in part, by NIH Grant CA71508 and ACS Grant CB202 (A. W.) and the Susan Komen Foundation for Breast Cancer Research (A.T.R., V.K.H., E.D.E.L.L.)

References

1. Lippman SM, Kessler JF and Meyskens FL Jr, Retinoids as preventive and therapeutic anticancer agents (Part II). *Cancer Treat Rep* **71:** 493–515, 1987.
2. Lippman SM, Kessler JF and Meyskens FL Jr, Retinoids as preventive and therapeutic anticancer agents (Part I). *Cancer Treat Rep* **71:** 391–405, 1987.
3. Lotan R, Suppression of squamous cell carcinoma growth and differentiation by retinoids. *Cancer Res* **54**(Suppl 7)**:** 1987s–1990s, 1994.
4. Lippman SM, Kavanagh JJ, Paredes-Espinoza M, Delgadillo-Madrueño F, Paredes-Casillas P, Hong WK, Massimini G, Holdener EE and Drakoff IH, 13-*cis*-Retinoic acid plus interferon-α2a in locally advanced squamous cell carcinoma of the cervix. *J Natl Cancer Inst* **85:** 499–500, 1993.
5. Lotan R, Retinoids in cancer chemoprevention. *FASEB J* **10:** 1031–1038, 1996.
6. Dmitrovsky E and Bosl GJ, Active cancer therapy combining 13-*cis*-retinoic acid with interferon-α. *J Natl Cancer Inst* **84:** 218–219, 1992.
7. Lippman SM, Heyman RA, Kurie JM, Benner SE and Hong WK, Retinoids and chemoprevention: Clinical and basic studies. *J Cell Biochem Suppl* **22:** 1–10, 1995.
8. Tallman MS and Wiernik PH, Retinoids in cancer treatment. *J Clin Pharmacol* **32:** 868–888, 1992.
9. Khuri FR, Lippman SM, Spitz MR, Lotan R and Hong WK, Molecular epidemiology and retinoid chemoprevention of head and neck cancer. *J Natl Cancer Inst* **89:** 199–211, 1997.
10. Kim JW, Kim YT, Choi SM, Kim DK and Song CH, Effect of 13-*cis*-retinoic acid with neoadjuvant chemotherapy in patients with squamous cervical carcinoma. *Am J Clin Oncol* **19:** 442–444, 1996.
11. Chambon P, A decade of molecular biology of retinoic acid receptors. *FASEB J* **10:** 940–954, 1996.
12. Boehm MF, Zhang L, Zhi L, McClurg MR, Berger E, Wagoner M, Mais DE, Suto CM, Davies PJA, Heyman RA and Nadzan AM, Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *J Med Chem* **38:** 3146–3155, 1995.
13. Gottardis MM, Bischoff ED, Shirley MA, Wagoner MA, Lamph WW and Heyman RA, Chemoprevention of mammary carcinoma by LGD1069 (Targretin): An RXR-selective ligand. *Cancer Res* **56:** 5566–5570, 1996.
14. Miller VA, Benedetti FM, Rigas JR, Verret AL, Pfister DG, Straus D, Kris MG, Crisp M, Heyman R, Loewen GR, Truglia JA and Warrell RP, Initial clinical trial of a selective retinoid X receptor ligand, LGD1069. *J Clin Oncol* **15:** 790–795, 1997.
15. Lehmann JM, Jong L, Fanjul A, Cameron JF, Lu XP, Haefner P, Dawson M and Pfahl M, Retinoids selective for retinoid X receptor response pathways. *Science* **258:** 1944–1946, 1992.
16. Teng M, Duong TT, Klein ES, Pino ME and Chandraratna RAS, Identification of a retinoic acid receptor α subtype specific agonist. *J Med Chem* **39:** 3035–3038, 1996.
17. Liaudet-Coopman EDE, Berchem GJ and Wellstein A, *In vivo* inhibition of angiogenesis and induction of apoptosis by retinoic acid in squamous cell carcinoma. *Clin Cancer Res* **3:** 179–184, 1997.
18. Wu D, Kan M, Sato GH, Okamoto T and Sato JD, Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. *J Biol Chem* **266:** 16778–16785, 1991.
19. Yoshida M, Kijima M, Akita M and Beppu T, Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem* **265:** 17174–17179, 1990.
20. Czubayko F, Liaudet-Coopman EDE, Aigner A, Tuveson AT, Berchem G and Wellstein A, A secreted FGF-binding protein can serve as the angiogenic switch for human cancer. *Nat Med* **3:** 1137–1140, 1997.
21. Czubayko F, Smith RV, Chung HC and Wellstein A, Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. *J Biol Chem* **269:** 28243–28248, 1994.
22. Liaudet-Coopman EDE and Wellstein A, Regulation of gene expression of a binding protein for fibroblast growth factor by retinoic acid. *J Biol Chem* **271:** 21303–21308, 1996.
23. Harris VK, Liaudet-Coopman EDE, Boyle BJ, Wellstein A and Riegel AT, Phorbol ester-induced transcription of a FGF-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. *J Biol Chem* **273:** 19130–19139, 1998.
24. Zhang X-K, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner J, Hermann T, Tran P and Pfahl M, Homodimer formation of retinoid X receptor induced by 9-*cis* retinoic acid. *Nature* **358:** 587–591, 1992.
25. Lee H-Y, Sueoka N, Hong W-K, Mangelsdorf DJ, Claret FX and Kurie JM, All-*trans*-retinoic acid inhibits Jun N-terminal kinase by increasing dual-specificity phosphatase activity. *Mol Cell Biol* **19:** 1972–1980, 1999.
26. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK and Rosenfeld MG, A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85:** 403–414, 1996.
27. Westin S, Kurokawa R, Nolte RT, Wisely GB, McInerney EM, Rose DW, Milburn MV, Rosenfeld MG and Glass CK, Interactions controlling the assembly of nuclear receptor heterodimers and co-activators. *Nature* **395:** 199–202, 1998.
28. Zou C-P, Clifford C, Xu X-C, Sacks PG, Chambon P, Hong WK and Lotan R, Modulation by retinoic acid (RA) of squamous cell differentiation, cellular RA binding proteins and nuclear RA receptors in human head and neck squamous cell carcinoma cell lines. *Cancer Res* **54:** 5479–5487, 1994.
29. Mangelsdorf DJ and Evans RM, The RXR heterodimers and orphan receptors. *Cell* **83:** 841–850, 1995.