Regulation of Gene Expression of a Binding Protein for Fibroblast Growth Factors by Retinoic Acid*

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Retinoids are potent regulators of growth and differentiation and have shown promise as chemotherapeutic agents against selected cancers in particular squamous cell carcinoma (SCC). Earlier studies from our laboratory showed that a secreted binding protein for fibroblast growth factors (BP) is expressed at high levels in SCC cell lines and tissue samples. Here we investigate whether retinoids affect BP gene expression in SCC. In six different human SCC cell lines, we found that all-trans-retinoic acid (tRA) down-regulated BP mRNA by 39–89% within 24 h. From this group of cell lines, we selected the ME-180 cell line for more detailed studies of the mechanisms of this regulation. tRA down-regulated BP mRNA in a time- and dose-dependent manner. The effect of tRA was reversible, and BP mRNA returned to control levels within 24 h after removal of tRA. We also measured BP mRNA half-life and performed nuclear run-on experiments to study if tRA down-regulates BP by destabilizing the mRNA and/or by decreasing the rate of transcription. BP mRNA in ME-180 cells is very stable with a half-life of >16 h, and tRA decreased BP mRNA with a half-time of 5 h. Actinomycin D and cycloheximide blocked the tRA effect, suggesting that transcriptional regulation as well as de novo protein synthesis contribute to this post-transcriptional regulation of BP mRNA levels. In addition, tRA decreased the rate of BP gene transcription by 2- to 3-fold within 1 h. We conclude that retinoids down-regulate BP gene expression by post-transcriptional as well as by transcriptional mechanisms.

The mechanisms by which retinoids suppress carcinogenesis and regulate differentiation and the expression of the transformed phenotype in SCCs have not been elucidated. It is thought that nuclear retinoid receptors act as ligand-activated trans-acting factors that mediate the effects of retinoids on gene expression and thereby alter the growth and differentiation of normal and tumor cells. Most studies have stressed modulation of differentiation, examining markers such as transglutaminase type I, loricrin, involucrin, filaggrin, and keratin K1 (11).

Recently, we found that tRA inhibits in vivo progression of a human SCC model system (ME-180 cells), and we hypothesized that the tRA action was through an inhibition of stromal cell-induced angiogenesis as well as stimulation of tumor apoptosis. The mechanism of the anti-angiogenic action of tRA in vivo is not known, but it could be due to the inhibitory effect on endothelial cell proliferation as well as to the production or release of angiogenic factors by tumor cells. Although it has been described that tRA decreased the transcription rate of epidermal growth factor receptor in ME-180 cells (12), classical angiogenic growth factors, such as FGFs, or vascular endothelial cell growth factor or their receptors have not been reported to be down-regulated by retinoids. Another possibility could be that tRA down-regulates auxiliary proteins required for the mechanism of action of angiogenic factors. A potential candidate could be a binding protein for FGFs (BP) that has been shown to positively modulate the activity of FGFs (13), and the present study reports on the regulation of BP gene expression by tRA.

BP is a secreted protein that binds to aFGF and bFGF in a non-covalent reversible manner (14). BP mRNA has been found to be expressed at high levels in SCC from patients and in SCC cell lines of different origin. We showed that expression of BP in a non-tumorigenic human cell line (SW-13), which expresses bFGF, leads to a tumorigenic and angiogenic phenotype of these cells (13). Moreover, expression of BP in these cells obviously solubilizes their endogenous bFGF from its extracellular storage and allows it to reach its receptor suggesting that BP serves as an extracellular carrier molecule for bFGF. Our current results show that BP expression is rapidly reduced by tRA and that both post-transcriptional and transcriptional events combine to regulate BP abundance in tRA-treated SCC cells.

MATERIALS AND METHODS

Cell Lines—Squamous cell carcinoma cell lines ME-180, FaDu, A431, SCC25, NCI-H596, and SW900 were obtained from the American Type Culture Collection. Cells were cultured in improved minimal essential medium (IMEM) (Biofluids, Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.).

Northern Analysis of mRNA—ME-180 cells were grown to 80% confluence on 150-mm tissue culture dishes, washed twice in serum-free IMEM, and then treated with tRA (Ligand Pharmaceuticals Inc., San Diego, CA) in serum-free IMEM. Total RNA was isolated with the RNA...
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**Fig. 1.** Effect of tRA on BP mRNA expression in squamous cell carcinoma cell lines. ME-180, FaDu, A431, SCC25, NCI-H596, and SW900 SCC cell lines were treated for 24 h with 10⁻⁵ m tRA. Total RNA was extracted, and Northern blot analyses were performed with 30 μg of total RNA/lane as described under “Materials and Methods.” After electrophoresis, the RNA was transferred to nylon membranes that were first hybridized with the BP probe, stripped, and then rehybridized with the GAPDH probe. Bands corresponding to BP mRNA (1.2 kb) and the control gene GAPDH mRNA (1.3 kb) are indicated. Signal intensities were quantified by phosphoimaging and normalized to the GAPDH control gene. A representative Northern blot from two to four separate experiments is shown in panel A, and the quantitation is in panel B.

STAT-60 method using commercially available reagents and protocols (Tel-Test Inc., Friendswood, TX). 30 μg of total RNA were separated by electrophoresis in 1.2% formaldehyde-agarose gel and then blotted onto nylon membranes (Schleicher & Schuell). The blots were prehybridized in 6 × SSC (0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 5 × Denhardt’s solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 100 μg/ml sonicated salmon sperm DNA (Life Technologies, Inc.) for 4 h at 42°C. Hybridization was carried out overnight at 42°C in the same buffer. After hybridization, blots were washed three times with 2 × SSC and 0.1% SDS for 10 min at 42°C and once with 1 × SSC and 0.1% SDS for 20 min at 65°C. Autoradiography was performed by intensifying screens at 70°C. Blots were stripped by boiling 2 × for 10 min in 1 × SSC and 0.1% SDS. Hybridization probes were prepared by random-primed DNA labeling (Boehringer Mannheim) of purified insert fragments from human BP (13), human RARγ, and RARβ (Ligand Pharmaceuticals Inc.) and human GAPDH (CLONTECH). The final concentration of the labeled probes was always greater than 2 × 10⁶ cpm/ml of hybridization solution. Quantification of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

Nuclear Run-on Analysis—ME-180 cells were grown to 80% confluance on 150-mm tissue culture dishes. Cells were treated with 10⁻⁵ m tRA in serum-free IMEM, and nuclei from 10⁷ cells for each time point were isolated after incubation in lysis buffer containing 0.5% Nonidet P-40 as described in Ref. 15. Nuclear run-on experiments were performed with [α-³²P]-UTP (Amersham Corp.). Equal amounts of radioactivity (0.5–1 × 10⁷ cpm) were hybridized to nitrocellulose filters containing 3 μg of each plasmid. After hybridization for 4 days at 42°C, the filters were washed four times with 2 × saline/sodium phosphate/EDTA, 0.1% SDS for 5 min at 25°C and treated for 30 min at 25°C in 2 × saline/sodium phosphate/EDTA containing 20 μg/ml RNase A. The filters were then washed four times for 30 min in 1 × saline/sodium phosphate/EDTA, 1% SDS at 65°C. The amount of radioactivity present in each slot was determined using a PhosphorImager after overnight exposure, and autoradiograms were exposed for 1–3 days with intensifying screens.

**RESULTS**

Down-regulation by Retinoic Acid of BP mRNA in SCCs—To study regulation of BP gene expression by retinoids in SCC, six human SCC cell lines were treated with tRA (10⁻⁵ m) for 24 h. As demonstrated in the Northern blots in Fig. 1, tRA decreased BP mRNA levels. PhosphorImager analysis showed that the reduction was by 79, 73, 83, 76, 89, and 39% in ME-180, FaDu, A431, SCC25, NCI-H596, and SW900 cells, respectively. The standard error of these experiments was less than 10% (n = 2–4 experiments/cell line). GAPDH mRNA remained unaffected by tRA treatment, as judged relative to the total amount of RNA loaded and was used to standardize the different mRNAs analyzed. Alternatively, β-actin was used. These data show that the tRA-induced down-regulation of BP mRNA is a general phenomenon in human SCC cell lines although the extent appears to differ.

We selected ME-180 cells for more detailed studies into the mechanisms of regulation of BP gene expression. The time course and dose dependence of tRA-induced down-regulation of BP mRNA in ME-180 cells are shown in Figs. 2 and 3. tRA (10⁻⁵ m) induced an 80% decrease in the steady-state level of
BP mRNA after 24 h (Fig. 2). The maximum effect was reached 8–12 h after tRA exposure with a half-time of 5 h for this effect and remained constant in the continuous presence of tRA for another 24 h. As a positive control gene, we used RARγ (16). As shown in Fig. 2, tRA rapidly induced a 2-fold increase in the steady-state level of RARγ mRNA. In contrast, RARα and RARβ mRNA levels were not modulated by tRA treatment (data not shown). The decrease of the BP mRNA was dependent on the concentration of tRA (Fig. 3). An 80% decrease in the steady-state level of BP mRNA was observed in cells grown for 24 h in the presence of 10 μM tRA, and we estimate the half-maximal effective concentration as 200 nM tRA. Consistent with previous reports, we observed no toxicity of tRA (10−2 M) on ME-180 cells (12).

We then studied the reversibility of the tRA effect on BP mRNA (Fig. 4). ME-180 cells were incubated for 24 h with tRA (10−5 M), and at 0, 8, 16, and 24 h after removal of tRA, RNA was isolated. BP mRNA levels increased from 20 to 71% of control value by 8 h and reached control levels by 24 h after removal of tRA. These data show that tRA-induced down-modulation of BP mRNA is a reversible phenomenon.

Effects of Actinomycin D and Cycloheximide on the Down-regulation of BP mRNA—The tRA-induced decrease in BP mRNA steady-state levels could be the result of a post-transcriptional or a transcriptional effect or a combination of both. We first assessed whether tRA treatment affected the stability of the BP mRNA. For that, experiments were performed to determine whether the addition of inhibitors of transcription (actinomycin D) or translation (cycloheximide) could inhibit the tRA-induced modulation of BP mRNA. Actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) was added without or with tRA (10−5 M), and BP mRNA levels were determined at various time points (Figs. 5 and 6). As can be seen in Fig. 5, the BP mRNA level did not change within 16 h after the addition of actinomycin D. To assure that the concentration of actinomycin D was sufficient to block transcription, we probed the same blots for two mRNA species with a relatively short half-life, i.e. RARβ and RARγ (17). Both mRNA levels were found to decrease after the actinomycin D treatment (see Fig. 5) as expected after transcription blockade. Furthermore, these data show that BP mRNA is relatively stable in ME-180 cells with a half-life of over 16 h. Simultaneous addition of tRA and actinomycin D completely blocked the effects of tRA (Fig. 5) as did cycloheximide (Fig. 6). This indicates that transcription as well as new protein synthesis are necessary for the tRA-induced down-regulation of BP mRNA.

To test whether a short time of tRA treatment would be sufficient to bring about degradation of BP mRNA, ME-180 cells were pretreated for 4 h with 10−5 M tRA and then actinomycin D was added to further inhibit transcription (Fig. 7). In comparison with tRA treatment alone (cf. Figs. 2 and 5), the addition of actinomycin D after tRA pretreatment did not affect the further decline of BP mRNA. This suggests that short term control of BP mRNA levels by tRA is predominantly regulated via mRNA stability.

Effects of tRA on BP Transcription Rates—To determine whether tRA also affects the rate of transcription of the BP gene, we next performed nuclear run-on experiments. Nuclei were isolated from ME-180 cells treated with tRA 10−5 M for 1,
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In the embryo, retinoic acid has been implicated as the natural morphogen responsible for pattern formation in developing chick limb buds (18), acting via nuclear hormone receptors of the steroid and thyroid hormone receptor superfamilies. These receptors have been thought to act primarily through direct modulation of gene transcription (19), but additional evidence has also emerged that ligands for these receptors are capable of influencing steady-state mRNA levels through post-transcriptional mechanisms, mainly through mRNA stabilization (20). Glucocorticoids stabilize human growth hormone mRNA (21); estrogens stabilize the low density lipoprotein II and vitellogenin mRNAs (22); and retinoic acid stabilizes the keratin 19 mRNA (23), c-fos mRNA (24), and proteolipid protein mRNA (25). However, there are examples of mRNAs whose stability is decreased by these hormones. Estrogens accelerate the turnover of albumin mRNA in Xenopus liver (26) and estrogen receptor mRNA in human mammary adenocarcinoma cells (27). It has also been reported that glucocorticoids increase the turnover of c-myc mRNA (28) and thymidine kinase (Tk-1) (29). Retinoic acid has been shown to destabilize adipsin mRNA (30), tyrosine aminotransferase mRNA (31), and myeloblastin mRNA (32).

In this report, we show that tRA rapidly down-regulates BP mRNA. This BP mRNA modulation is a general feature since it was observed in six SCC cell lines. The tRA-induced down-regulation of BP mRNA in the ME-180 cell is due to a combined decrease in the stability of its mRNA and of the transcriptional rate of the gene. Our data show that BP mRNA is generally very stable in ME-180 cells. We were not able to precisely determine the half-life of BP mRNA because blockade of transcription by exposure of cells to actinomycin D for longer than 16 h leads to general cytotoxicity. Clearly, the half-life of BP mRNA is longer than 16 h, and we conclude that the early down-regulation of steady-state BP mRNA by tRA is mainly due to increased turnover of the BP mRNA. Actinomycin D and cycloheximide block the effects of tRA when either inhibitor is added simultaneously with tRA. Furthermore, once tRA has been allowed to act for 4 h, the addition of actinomycin D no longer affects the decline of BP mRNA brought about by the initial treatment with tRA. Since parallel addition of actinomycin D and of tRA blocked the down-regulation of BP mRNA, the data also imply that tRA rapidly induces transcription of a gene product that decreases the stability of BP mRNA. The
data preclude the alternative possibility that tRA inhibits the expression of a gene product that is required to stabilize a generically unstable BP mRNA. If the latter were the case, one would expect any nonspecific inhibitor of transcription or translation (e.g. actinomycin D and cycloheximide) to accelerate the degradation of BP mRNA due to the reduction of the stabilizing gene product. Reversibility of the tRA effect within 24 h after removal of the drug furthermore indicates that the product induced has a relatively short half-life.

Several studies have provided some insight into the nature of mRNA decay (33) and have identified structural features that determine susceptibility to decay, and we wondered whether BP mRNA would contain any signature sequence that may confer regulatability of RNA stability. The selectivity of mRNA decay can be best explained by the action of specific factors, acting in trans, that recognize unique cis-elements in the mRNA. Endonucleolytic cuts triggered by interactions between trans-acting factors and cis-elements result in rapid mRNA destruction. A specific sequence promoting mRNA decay (5'-AUUUA-3') has been identified in the 3'-noncoding regions of a variety of mRNAs (34). The manner in which this sequence promotes mRNA decay is unknown although it may be recognized and cleaved by a specific endonuclease. Recent studies identified functionally independent determinants within the c-fos transcript that specifically target this message for rapid decay (24). One of the determinants is this AU-rich element that is present in the 3'-untranslated region of c-fos mRNA. A second c-fos instability determinant, which is located in the protein coding region of the c-fos message, is structurally unrelated to this element. Interestingly, we found that an AU-rich element is also present in the 3'-noncoding sequence of human BP mRNA, and we speculate that this sequence might be regulating stability.

Our experiments also provide evidence for transcriptional down-regulation of BP in addition to the post-transcriptional effects. We show that the decrease in BP transcription begins very early, 1 h following tRA addition (Fig. 8). From the relatively fast kinetics of tRA-mediated effects on BP gene expression, it is tempting to speculate that the response may be controlled by a direct interaction between a retinoic acid receptor and transcriptional regulatory sequences in the BP gene. However, the fast negative response of the transcription rate to a nuclear receptor ligand does not necessarily imply a direct interaction between receptor and promoter, but it may be in parallel with the negative regulatory mechanism, e.g. described for the interaction between the glucocorticoid receptor and AP1 transcription factors (35). On the other hand, our findings could also be explained by the existence of a negative tRA response element in the BP promoter as shown for other genes (36–38).
In conclusion, tRA down-regulates BP gene expression in different SCC cell lines, and more detailed studies with a representative cell line show that this down-regulation occurs both at the transcriptional and post-transcriptional levels. Interestingly, the tRA effects on BP mRNA stability are sensitive to actinomycin D and cycloheximide, suggesting that tRA-induced gene transcription and protein synthesis are required for the destabilizing effect on BP mRNA.

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