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Research Article

Suppression of Mammary Carcinoma Cell Growth by Retinoic Acid: the Cell Cycle Control Gene *Btg2* Is a Direct Target for Retinoic Acid Receptor Signaling

Leslie J. Donato, Jean H. Suh, and Noa Noy

Division of Nutritional Sciences, Cornell University, Ithaca, New York

Abstract

The anticarcinogenic activities of retinoic acid (RA) are believed to be mediated by the nuclear RA receptor (RAR) and by the RA-binding protein cellular RA-binding protein-II (CRABP-II). In MCF-7 mammary carcinoma cells, growth inhibition by RA entails an early cell cycle arrest followed by induction of apoptosis. Here, we aimed to obtain insights into the initial cell cycle response. We show that a 3- to 5-h RA pulse is sufficient for inducing a robust growth arrest 2 to 4 days later, demonstrating inhibition of the G_1-S transition by RA is triggered by immediate-early RAR targets and does not require the continuous presence of the hormone throughout the arrest program. Expression array analyses revealed that RA induces the expression of several genes involved in cell cycle regulation, including the p53-controlled antiproliferative gene B-cell translocation gene, member 2 (*Btg2*) and the BTG family member *Tob1*. We show that induction of *Btg2* by RA does not require *de novo* protein synthesis and is augmented by overexpression of CRABP-II. Additionally, we identify a RA response element in the *Btg2* promoter and show that the element binds retinoid X receptor/RAR heterodimers *in vitro*, is occupied by the heterodimers in cells, and can drive RA-induced activation of a reporter gene. Hence, *Btg2* is a novel direct target for RA signaling. In concert with the reports that Btg2 inhibits cell cycle progression by down-regulating cyclin D1, induction of *Btg2* by RA was accompanied by a marked decrease in cyclin D1 expression. The observations thus show that the antiproliferative activity of RA in MCF-7 cells is mediated, at least in part, by Btg2. [Cancer Res 2007;67(2):609–15]

Introduction

The vitamin A metabolite retinoic acid (RA) plays key roles in embryonic development and in tissue remodeling in the adult. This hormone also displays distinct anticarcinogenic activities and is currently used and being tested in clinical trials as a therapeutic and preventive agent in several types of cancer (1–3). These pleiotropic activities are exerted primarily through the ability of RA to regulate gene expression and are mediated by the nuclear hormone receptors termed RA receptors (RAR). RARs associate with the retinoid X receptor (RXR) to form heterodimers that bind to RAR response elements (RARE) in regulatory regions of target genes and enhance transcriptional rates upon binding of RA (4–6). The transcriptional activities of RA are also mediated by a small soluble protein termed cellular RA-binding protein II (CRABP-II; refs. 7, 8). Upon binding of RA, CRABP-II mobilizes from the cytosol to the nucleus where it directly "channels" RA to RAR, thereby facilitating delivery of RA to the receptor and enhancing its transcriptional activity (9–11). Consequently, ectopic expression of CRABP-II sensitizes cultured mammary carcinoma cells to the growth inhibitory effects of RA and suppresses the tumor development in two mouse models of breast cancer (10, 12).

The pathways that underlie the anticarcinogenic activities of RA vary between different cell types. RA induces a G_1-G_0 growth arrest in embryonic teratocarcinoma F9 cells (13), differentiation in myeloid HL-60 cells (14), and apoptosis in NB4 acute promyelocytic leukemia cells (15). However, information on direct target genes that mediate the antiproliferative activities of RAR remains scarce. It was reported that RA-induced apoptosis and differentiation in NB4 promyelocytic leukemia cells involve ubiquitin-activating enzyme E1-like protein, CCAAT/enhancer binding protein ε, and tumor necrosis factor–related apoptosis-inducing ligand (16–18). Several genes (i.e., *Bcl-2, survivin*, the tumor suppressor gene *PDCD4*, the transcription factor *SOX9*, and, importantly, *cyclin D1*) were reported to be down-regulated in conjunction with RA-induced growth inhibition in breast cancer cells (19–24). However, as liganded RAR usually functions as a transcriptional activator, these genes are unlikely to comprise direct targets, and the mechanisms by which RA regulates their expression are unknown. Recently, we showed that RA-induced apoptosis in the mammary carcinoma MCF-7 cells is associated with up-regulation of the expression of the proapoptotic genes *caspase-7* and *caspase-9* (11) and with increased activation of these serine proteases. Although *caspase-7* seems to be an indirect responder, *caspase-9* was found to be a direct target for RAR and is thus an important mediator of RA-induced apoptosis in these cells.

Notably, inhibition of MCF-7 cell growth by RA seems to be a multifaceted process involving the induction of both apoptosis and cell cycle arrest (25–27). The present study was undertaken to obtain insights into the initial cell cycle response, identify direct RAR target genes that are involved in this response, and examine the contribution of CRABP-II to the process.

Materials and Methods

Cells. MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

RA was purchased from Calbiochem (La Jolla, CA).

Antibodies. Rabbit IgG, pan-RAR, RARα, and RXRα were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against mCRABP-II (5CRA3B3) were a gift from Pierre Chambon. Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase–conjugated antibodies were from Amersham (Arlington Heights, IL).

Note: L.J. Donato and J.H. Suh contributed equally to this work.

Requests for reprints: Noa Noy, Division of Nutritional Sciences, Cornell University, 222 Savage Hall, Ithaca, NY 14853. Phone: 607-255-2490; E-mail: nn14@cornell.edu.

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Proteins. Recombinant histidine-tagged proteins were expressed in *Escherichia coli* and purified as previously described (28). Purified proteins were dialyzed against HEK buffer [10 mmol/L HEPES (pH 8), 0.1 mmol/L EDTA, 100 mmol/L KCl] and stored at −20°C in 50% glycerol. CRABP-II-L28C was covalently labeled with the environmentally sensitive fluorescent probe fluorescein using the thiol-reactive 5-(bromomethyl)fluorescein (BMF; Molecular Probes, Eugene, OR). Protein was incubated with BMF at a protein/BMF mole ratio of 1:3 in HEK buffer (pH 7.3) for 2 h and dialyzed against HEK buffer containing 1 mmol/L DTT and 5% glycerol. Typically, the mole ratio of fluorescein to protein in the resulting labeled proteins was 0.7 to 1.

Reporter construct. The 95-bp oligonucleotide containing the putative B-cell translocation gene, member 2 (*Btg2*) RARE was synthesized with *Hin*dIII and *Bam*HI restriction site overhangs at 5′ and 3′ ends, respectively, and subcloned into the luciferase reporter vector.

Affymetrix expression array analyses were conducted as previously described (11). Iobion's GeneTraffic was used to perform robust multi-chip analysis and cluster genes with similar activity by summary function.

Fluorescence-activated cell sorting. MCF-7 cells were seeded in six-well plates (120,000 per plate) in DMEM supplemented with 10% FBS and grown overnight. Cells were treated with RA in DMEM containing 1% FBS and then incubated with 5′-bromo-2′-deoxyuridine (BrdUrd; Sigma, St. Louis, MO; 30 μg/mL, 20 min, 37°C). The medium was removed; cells were washed twice in PBS; trypsinized cells were collected; and cells were centrifuged. The pellet was resuspended in 1 mL PBS, rapidly injected into 10 mL 70% ethanol, and incubated overnight at 4°C. Cells were centrifuged and resuspended in 1 mL of 2 N HCl/0.5% Triton X-100. Following a 30-min incubation, cells were centrifuged, resuspended in 1 mL of 0.1 mol/L $Na_2B_4O_7 \cdot 10H_2O$ (pH 8.5) and collected, and 1 mL of dilution buffer (0.5% Tween 20 and 1% bovine serum albumin in PBS) was added. Anti-BrdUrd FITC antibody (Becton Dickinson, Mountain View, CA; 20 μL per 10^6 cells) was added, and suspensions were incubated at room temperature for 30 min. Stained cells were washed with 5 mL of dilution buffer and resuspended in 1 mL PBS containing propidium iodide (Sigma; 5 μg/mL) and analyzed by fluorescence-activated cell sorting.

Chromatin immunoprecipitation. MCF-7 cells were seeded in 100-mm plates (2,000,000 per plate) and grown to confluency. Proteins were cross-linked to DNA (10% formaldehyde, 20 min, 37°C), and the reaction was quenched by glycine (125 mmol/L, 5 min, 4°C). Cells were washed twice with PBS, scraped, lysed (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris at pH 7.9, 1 mmol/L DTT, and protease inhibitors; Roche, Indianapolis, IN), and incubated (4°C, 45 min). Lysed cells were sonicated to obtain DNA fragments of 300 to 700 bp. One fifth of samples were stored at 4°C as an input. The remaining sample was diluted [0.5% Triton X-100, 2 mmol/L EDTA, 2 mmol/L Tris (pH 7.9), 150 mmol/L NaCl, 1 mmol/L DTT, protease inhibitors, salmon sperm DNA] and precleared (1 h, 4°C) with protein A beads. Following centrifugation, supernatant was transferred; appropriate antibodies (3.5 μg) were added; and mixtures were incubated overnight (4°C). Protein A beads were added (2 h, 4°C); washed (3×) using a buffer containing 0.25% NP40, 0.05% SDS, 2 mmol/L EDTA, 20 mmol/L Tris (pH 8), 250 mmol/L NaCl, leupeptin, and aprotinin; and washed once in TE buffer. Beads were resuspended in 100 mmol/L $NaHCO_3$ containing 1% SDS and incubated to reverse the cross-link (4 h, 65°C). Proteins were digested with proteinase K (1 h), and DNA was purified (nucleotide removal kit, Qiagen, Chatsworth, CA). The putative RARE-containing region of *Btg2* was amplified by PCR (forward primer, 5′-CCCGGCTACACTGTATATTGACTTGG-3′; reverse primer, 5′-GGGTTTCAT-CACGTTGGTCAGGAT-3′).

Transactivation assays. MCF-7 cells were seeded in 12-well plates (75,000 per well) in DMEM containing 10% FBS and grown overnight. Cells were transfected using Fugene (Roche) in DMEM containing 1% FBS with tk-luciferase reporter vector containing the Btg2 RARE (400 μg) and pCH110 encoding β-galactosidase (300 μg). Following an overnight incubation, cells were treated with RA in serum-free DMEM for 24 h and lysed. Luciferase activity was measured using the luciferase assay system (Promega, Madison, WI) and corrected for transfection efficiency by β-galactosidase activity.

Quantitative real-time PCR. Total RNA was extracted using RNeasy (Qiagen), and cDNA was generated using GeneAmp RNA PCR (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was done in quadruplicates using Taqman chemistry and Assays on Demand probes (Applied Biosystems) for Btg2 (Hs00198887_m1) and cyclin D1 (Hs00277039_m1). 18S rRNA (4319413E-0312010) was used as a loading control. Analysis was carried out using the relative standard method (Applied Biosystems Technical Bulletin No. 2).

Electrophoretic mobility shift assay. The 95-bp oligonucleotide containing *Btg2*-RARE was end-labeled with [^{32}P]dCTP by filling in fragments with Klenow, and free nucleotides were removed (nucleotide removal kit, Qiagen). The labeled probe (∼1 ng) was incubated with appropriate receptors (100 nmol/L, 20 min) in HEDGK buffer [10 mmol/L HEPES (pH 8), 0.1 mmol/L EDTA, 0.4 mmol/L DTT, 100 mmol/L KCl, 15% glycerol] in the absence or presence of unlabeled competitor DNA or a 95-bp oligonucleotide of the mutated sequence GGAGGGcgAGGGGGagA-GAGGG. Protein-DNA complexes were resolved on 5% polyacrylamide gel and visualized by autoradiography.

Measurements of RA concentration in cell lysates. MCF-7 cells were seeded in 60-mm dishes (350,000 per plate) in DMEM containing 10% FBS. Cells were washed with PBS and treated with RA added in serum-free DMEM. Media were removed; cells were washed with PBS; and media were replaced with DMEM. Cells were washed and collected in 1 mL PBS and pelleted, and ligand was extracted by resuspension in 100% ethanol. Cell debris was centrifuged, and ethanol was collected and used in subsequent determinations. Pellets were dissolved in 1 mol/L NaOH (5 h), and protein content was measured by Bradford assay (Bio-Rad, Richmond, CA). RA concentrations in ethanol extracts were measured essentially as described (29). Briefly, bacterially expressed CRABP-I-L28C was purified and labeled with the fluorescent probe fluorescein, and the labeled protein was used as a "readout" for its ligand. Standard curves were made by extracting untreated MCF-7 cells into ethanol containing known concentrations of RA. Fluorescein-labeled-CRABP-I-L28C (160 nmol/L) was titrated with the various extracts, and the progress of the titrations was monitored at $\lambda_{excitation}/\lambda_{emission}$ of 491 nm/519 nm.

Results

MCF-7 cells arrest in G_1 in response to RA. MCF-7 cells were treated with RA (1 μmol/L) for 24 or 72 h, and the cell cycle distribution was examined using a BrdUrd incorporation assay. Cells were also stained with propidium iodide, and the fraction of cell populations in different cell cycle phases was determined by flow cytometry. The data (Fig. 1*A*) showed that RA treatment resulted in a marked increase in the cell population in the G_1 phase, leading to an overall 1.8-fold increase at 72 h. The increase was accompanied by a corresponding decrease in residency in S and in G_2-M phases, showing that RA inhibits the G_1-S transition. In addition to a marked effect on cell cycling, RA also triggered DNA fragmentation, reflected by an increase of the fraction of cells in the sub-G_1 population. In accordance with a G_1 arrest, the expression level of cyclin D1 mRNA decreased by ∼2-fold upon 24 h of RA treatment (Fig. 1*B*).

Rate of degradation of RA in MCF-7 cells. The biological activities of RA are believed to be mediated by induction of expression of RAR target genes. However, whereas RA-triggered changes in gene expression can be observed within several hours, cell cycle arrest in MCF-7 cells is observed 1 to 3 days following treatment (Fig. 1), and an ultimate apoptotic response is manifested 5 to 7 days later (11). To examine whether a short-term treatment with RA is sufficient for triggering a subsequent cell cycle arrest, or whether the response requires the continuous presence of the ligand, MCF-7 cells were treated with RA; the ligand was removed from the medium; and cellular responses were

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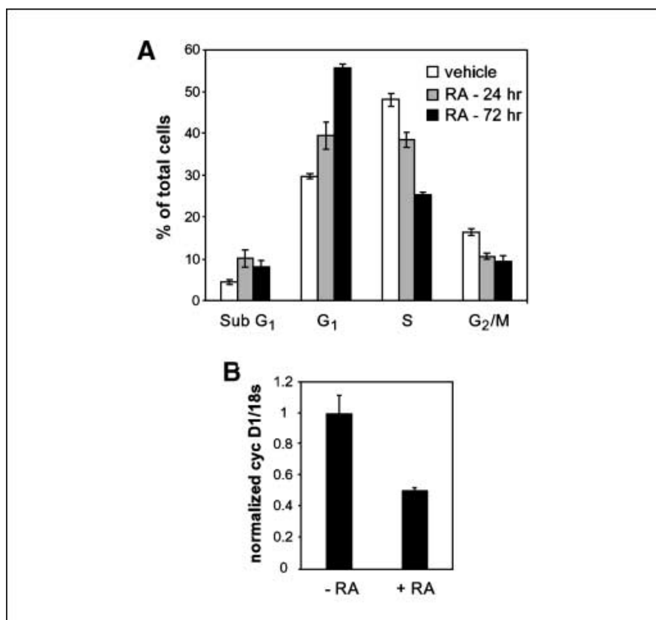

Figure 1. RA reduces the expression of cyclin D1 and induces a G_1 arrest in MCF-7 cells. *A,* MCF-7 cells were treated with vehicle or RA (1 μmol/L) for 24 or 72 h before incubation with BrdUrd (20 min). Cells were then fixed in ethanol, stained with FITC-conjugated anti-BrdUrd antibodies and with propidium iodide, and analyzed by fluorescence-activated cell sorting. *B,* MCF-7 cells were treated with vehicle or RA (1 μmol/L) for 24 h, total RNA was extracted, and cyclin D1 mRNA was measured by quantitative real-time PCR. Values were normalized to 18S rRNA level, and fold activation is presented. *Columns,* mean (n = 3); *bars,* SD.

monitored. To assess whether removal of RA from the medium indeed results in rapid depletion of the RA stores of the cell, the half-life of RA in MCF-7 cells was measured.

The concentrations of RA in MCF-7 cells were measured using our recently described fluorescence-based method (29). This method uses CRABP-I, a protein that binds RA with strict selectivity and high binding affinity, as a "readout" for this ligand. A CRABP-I mutant in which L28 has been replaced with a cysteine is covalently labeled with the fluorescent probe fluorescein. Ligand binding by the labeled protein leads to a decrease in the fluorescence of the probe, enabling direct measurements of RA concentration (Fig. 2*A*). A calibration curve (Fig. 2*B*) is obtained by extracting nontreated cells using ethanol containing known amounts of RA and titrating labeled CRABP-I-L28C with the extracts to obtain initial slopes characterizing the response at each RA concentration. To measure the rate of degradation of RA in MCF-7 cells, cells were incubated with RA (1 μmol/L); the medium was removed; cells were washed; RA was extracted into ethanol; and the concentration of RA was measured. The amount of RA found to accumulate in the cells after 1 h of RA treatment was found to be 2 to 3 nmol/mg protein (range from three experiments). Following removal of RA from culture media, the concentration of RA in the cells decreased rapidly, with ~50% of the ligand disappearing within ~20 min (Fig. 2*C*). Interestingly, plotting the data on a log scale to extract the rate constant of the reaction revealed that RA degradation is comprised of a two-phase process (Fig. 2*C, inset*). These observations may reflect the existence of two independent degradation mechanisms: a rapid degradation process characterized by a low K_m and a high V_{max} and a second process displaying a high K_m and a low V_{max}. Alternatively, the behavior may reflect the existence of two intracellular RA pools that degrade at different rates. Regarding the latter alternative, it is possible that free RA degrades rapidly with a half-life of ~18 min, whereas protein-bound RA is metabolized at a slower rate characterized by a half-life of 112 min. To address the possibility that binding to CRABP retards the rate of degradation of RA, we examined the rate of disappearance of RA in MCF-7 cells in which the expression of CRABP-II was reduced by ~80% by using small interfering RNA technology. However, RA degradation in cells with reduced CRABP-II expression followed a similar pattern to that obtained in non-transfected cells (data not shown), suggesting that other mechanisms are responsible for the two-phase disappearance of RA in MCF-7 cells.

A short-term treatment with RA is sufficient to elicit cell cycle arrest. The effectiveness of a short-term exposure to RA in regulating cell cycle events was then examined. MCF-7 cells were either pulsed with RA for 3 to 5 h or continuously exposed to the hormone for the entire duration of the experiments, and cell cycle distribution was analyzed at 2 or 4 days following treatment initiation (Fig. 3). In cells pulsed with RA, a G_1 cell cycle arrest was

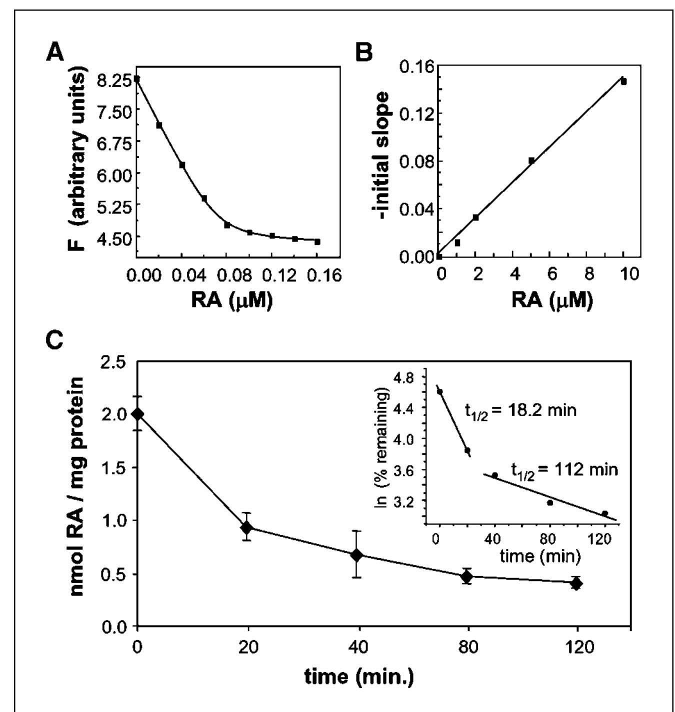

Figure 2. Rate of degradation of RA in MCF-7 cells. *A,* fluorescence titration of fluorescein-labeled CRABP-I-L28C. Protein (1 μmol/L) was titrated with RA, and the progress of the titration was followed by monitoring changes in the fluorescence of protein-bound probe ($\lambda_{excitation}$ = 492 nm; $\lambda_{emission}$ = 520 nm). Data were fitted to a binding equation (*line through points*). *B,* calibration curve. MCF-7 cells were cultured in 60-mm plates and extracted using ethanol (70 μL) containing known amounts of RA. Fluorescein-labeled CRABP-I-L28C (0.16 μmol/L) was titrated by consecutive additions of 5 μL of each standard solution, and fluorescence of the labeled protein was recorded. Absolute values of the initial slopes ($-\Delta F/\mu L$) of individual titrations were plotted as a function of the RA concentration in each standard to obtain a calibration curve. *C,* RA degradation in MCF-7 cells. Cells were treated with 1 μmol/L RA for 1 h; RA was removed from the media, and cells were extracted in ethanol at the indicated times. Fluorescein-labeled CRABP-I-L28C was titrated with each extract as described in (*A*), and the concentration of RA was obtained from initial slopes of titrations and the calibration curve. Data were normalized to the amount of cellular protein. *Bars*, SD. *Inset,* data plotted on a log scale to obtain the half-life of degradation.

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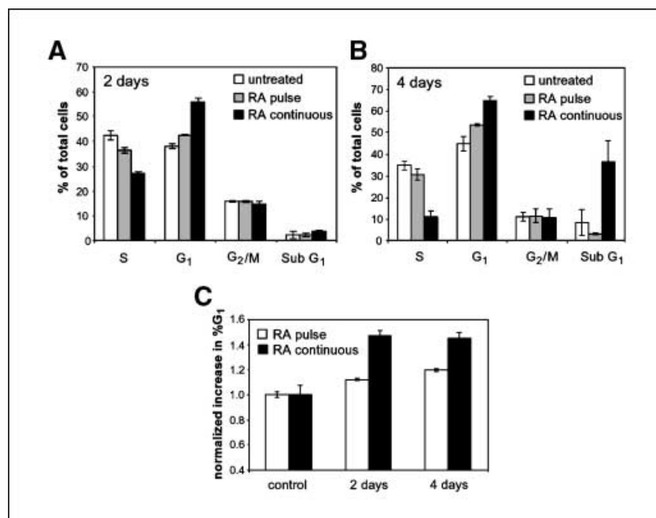

Figure 3. RA "pulse" is sufficient to trigger cell cycle arrest in MCF-7 cells. MCF-7 cells were treated with vehicle or RA (1 μmol/L) for several hours or for the entire duration of the experiment. Cells were incubated with BrdUrd, fixed, stained with FITC-conjugated antibodies against BrdUrd and with propidium iodide, and analyzed by flow cytometry. *A,* cells were treated with RA for 3 h (*RA pulse*) or for 2 d (*RA continuous*). *B,* cells were treated with RA for 5 h (*RA pulse*) or for 4 d (*RA continuous*). For continuous treatment, RA was replenished after 48 h. *C,* RA-induced increases in the fraction of cells in the G_1 phase, normalized to the fraction in G_1 of untreated cells. *Columns,* mean ($n = 4$); *bars,* SD.

evident after 2 days (Fig. 3*A*) and became more pronounced after 4 days (Fig. 3*B*). Hence, activation of initial targets of RA signaling seems to be sufficient for triggering a subsequent cell cycle arrest program in MCF-7 cells. Continuous treatment with RA resulted in a somewhat more enhanced arrest response and in induction of apoptosis, which became evident after day 4. It is worth noting, however, that the cell cycle response to the pulsed treatment seemed to be merely delayed rather than dampened (Fig. 3*C*). In contrast, a short-term exposure to RA was not sufficient to elicit an apoptotic response within the duration of the experiments.

RA up-regulates expression of cell cycle–regulating genes in MCF-7 cells. To identify genes that may mediate the RA-induced cell cycle arrest, an expression array analysis was done. MCF-7 cells were treated with vehicle or RA (50 nmol/L, 4 h), and total RNA was isolated. Probes were generated and hybridized to Affymetrix human U133 A/B arrays, monitoring >40,000 genes and expressed sequence tags. The differences in gene expression profiles between untreated and RA-treated cells were analyzed. Genes whose expression levels were up-regulated by RA were clustered by similar biological functions using the GeneTraffic software system (Iobion, La Jolla, CA).

Several genes that are known to be involved in cell cycle regulation were identified (Table 1). Among these, *Btg2* displayed a >2-fold increase in expression in response to RA. Btg2 (*Tis21, Pc3*, or *APRO1*) belongs to the antiproliferative (APRO) family and is known to function in regulating the G_1-S progression (30). Correspondingly, decreased Btg2 expression has been linked to cancerous states (31, 32). Expression of another BTG family member that functions as a negative cell cycle regulator, transducer of ERBB2 (*Tob1*), was also induced (33).

Btg2 is a direct target for transcriptional activation by RAR. To validate the array data, the effects of RA treatment on the mRNA expression levels of Btg2 and the related Tob1 were investigated. MCF-7 cells were treated with RA and lysed, and total RNA was extracted. cDNA was generated, and mRNA levels of Btg2 and Tob1 were measured by quantitative real-time PCR using Taqman probes. Data were normalized to 18S rRNA. In good agreement with the Affymetrix array data, RA treatment increased the level of Btg2 mRNA by close to 3-fold (Fig. 4*A*) and the level of mRNA for Tob1 by 1.8-fold (mean of two experiments that differed by 10%).

Regulation of expression of RA-responsive genes may be exerted directly (i.e., mediated by a RARE). Alternatively, responses may reflect secondary events involving RAR control of immediate target genes, which, in turn, are involved in downstream events leading to the observed modulation. Hence, an important question that arises is whether Btg2 comprises a direct target for RAR. This question is particularly pertinent considering the paucity of information on the mechanisms by which RA exerts its anticarcinogenic activities. To determine whether Btg2 is a direct target for RAR, the effect of cycloheximide treatment on Btg2 level was examined. Cycloheximide inhibits protein synthesis and will thus abolish secondary events that require *de novo* protein synthesis but will not affect direct transcriptional responses. Cells were pretreated with

Table 1. Genes involved in cell cycle regulation induced by RA in MCF-7 cells

Name	Abbreviation	Accession no.	Fold	Proposed function
BTG family, member 2	*Btg2*	NM_006763	2.30	Regulation of the G_1-S transition
Src family associated phosphoprotein 2	*SCAP2/SKAP55R*	NM_003930	1.39	Implicated in myeloid differentiation and growth arrest
Transducer of ERBB2, 1	*Tob1*	NM_005749	1.36	Negative cell cycle regulator (BTG family member)
Protein phosphatase 1B (formerly 2C), magnesium-dependent, β isoform	*PPM1B/PP2CB*	AJ271932	1.32	Dephosphorylation of cyclin-dependent kinases, leading to cell growth arrest or death
Max binding protein	*ROX/MNT*	NM_020310	1.30	Antagonist of Myc-dependent transcriptional activation and cell growth
abl-interactor 1	*Abi1/E3b1*	AF006516	1.29	Inhibits serum-induced proliferation of NIH3T3/EGFR cells, and v-Abl–mediated transformation of cultured cells
RAD17 homologue (*Schizosaccharomyces pombe*)	*FRP1/ATR/SCKL1*	U49844	1.26	Similar to *S. pombe* rad3, a cell cycle checkpoint gene required for cell cycle arrest and DNA damage repair

NOTE: MCF-7 cells were treated with vehicle or RA (50 nmol/L) for 4 h. Affymetrix expression array was used to compare the mRNA expression of untreated versus treated cells. Fold inductions of genes involved in cell cycle regulation are presented.

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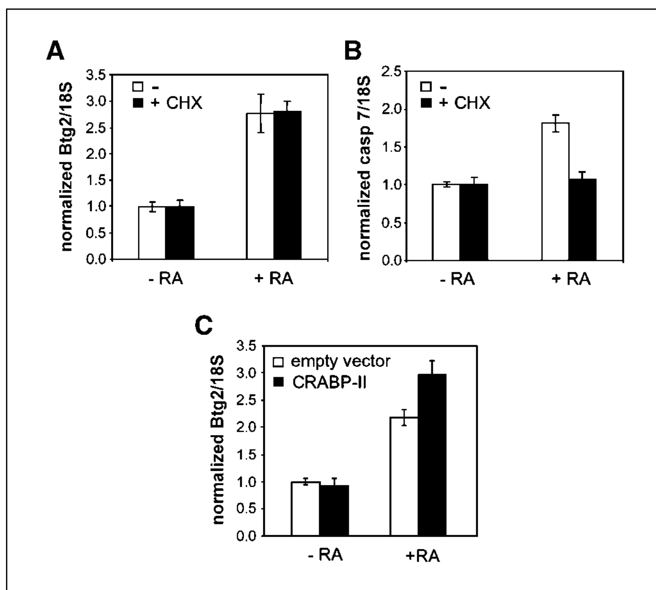

Figure 4. Btg2 is a direct target for RA signaling. *A,* MCF-7 cells were treated with cycloheximide (*CHX*; 20 μg/mL) for 10 min, followed by addition of RA (50 nmol/L). Cells were incubated for 30 min, total RNA was extracted, and Btg2 mRNA level was measured by quantitative real-time PCR and normalized to 18S rRNA. *B,* MCF-7 cells were treated with cycloheximide (20 μg/mL) for 10 min, followed by addition of RA (50 nmol/L). Following a 4-h incubation, total mRNA was extracted, and caspase-7 mRNA was measured by quantitative real-time PCR and normalized to 18S rRNA. *C,* MCF-7 cells were transfected with either an empty vector or a vector encoding CRABP-II. Cells were treated with vehicle or RA (50 nmol/L) for 4 h, total RNA was extracted, and Btg2 mRNA was measured by quantitative real-time PCR. Data were normalized to 18S rRNA, and fold activation is presented. *Columns,* mean (n = 3); *bars,* SD.

cycloheximide and then treated with RA (50 nmol/L, 4 h), and Btg2 mRNA expression levels were determined (Fig. 4*A*). As a control, we examined the effect of cycloheximide on the ability of RA to up-regulate the expression of *caspase-7*, a gene that has been previously shown to be an indirect target for RAR (Fig. 4*B*; ref. 11). The analysis showed that whereas up-regulation of the indirect target *caspase-7* was abolished upon cycloheximide treatment, inhibition of protein synthesis did not hinder the ability of RA to enhance the expression of *Btg2*. Hence, the observations show that *Btg2* is a direct target for RAR.

CRABP II augments RA-induced up-regulation of Btg2. We previously showed that the RA-binding protein CRABP-II directly delivers RA from the cytosol to RAR in the nucleus, thereby enhancing the transcriptional activity of the receptor (10, 11). As the data showed that Btg2 is a direct target for RAR signaling, it was of interest to examine whether up-regulation of its expression in response for RA is augmented by CRABP-II. To this end, MCF-7 cells were transfected with an expression vector encoding CRABP-II and treated with vehicle or RA. Measurements of the levels of Btg2 mRNA by quantitative real-time PCR (Fig. 4*C*) showed that ectopic overexpression of CRABP-II significantly enhanced the RA-induced up-regulation of the expression of the gene. Notably, CRABP-II overexpression alone did not increase the expression of Btg2, indicating that CRABP II does not regulate Btg2 expression independently of RA.

A functional RARE is present in the promoter of Btg2. Consensus RAREs consist of two direct hexameric repeats of the sequence PuG(G/T)TCA spaced by either 2 or 5 bp (DR2 and DR5). To identify the response element that might mediate the RA responsiveness of Btg2, a 4-kb stretch upstream of the transcription start site of the gene was screened for potential RAREs (TransFac).[1] Three DR2 half-sites were found to be present 3,250 bp upstream of the start site (Fig. 5*A*). This element, along with flanking sequences on both sides to a total of 95 bp, was generated and labeled with ^{32}P, and its ability to bind RXR-RAR heterodimers was examined by electrophoretic mobility shift assays. RAR and RXR lacking their NH_2-terminal A/B domain (RARαΔAB and RXRαΔAB) were used. The truncated proteins were used because of their greater yield upon bacterial expression compared with their full-length counterparts. The ligand-binding, heterodimerization, and DNA-binding properties of RARαΔAB and RXRαΔAB are indistinguishable from those of the full-length receptors and are thus appropriate for the assays. Proteins were bacterially expressed and purified and incubated with the 95-bp oligonucleotide containing the Btg2 RARE. Protein-DNA complexes were resolved by nondenaturing PAGE and visualized by autoradiography (Fig. 5*B*). Neither RAR nor RXR alone efficiently shifted the mobility of oligonucleotide. Addition of both receptors resulted in the appearance of a shifted band, reflecting binding of the heterodimer to the element. The observations that a cold probe containing the putative element effectively competed for binding to the RXR-RAR heterodimer, whereas the same 95-bp fragment mutated at the RARE did not, further showed the specificity of the interactions.

To determine if the RAR/RXR heterodimer binds to the Btg2 RARE in cells, chromatin immunoprecipitation assays were carried out. Proteins were cross-linked to chromatin in MCF-7 cells, sonicated, and immunoprecipitated using antibodies for RAR or RXR. A 200-bp region flanking the Btg2 RARE was amplified by PCR to detect receptor binding. The data (Fig. 5*C*) showed that antibodies against either RAR or RXR precipitated the putative Btg2 RARE, indicating that the element is occupied by the RXR-RAR heterodimer in cells.

To examine the functionality of the putative RARE, the 95-bp oligonucleotide containing the element was cloned upstream of a luciferase reporter; the reporter was transfected into MCF-7 cells; and transactivation assays were carried out. The data (Fig. 5*D*) showed a dose-responsive activation of reporter expression by RA, indicating that the element indeed comprises a functional RARE. The magnitude of the effect was not very large, suggesting that transcriptional regulation of Btg2 expression is likely to be mediated by additional elements. Nevertheless, RA responsiveness and the observations that mutation of the RARE abolished the response (Fig. 5*E*) further attest to its functionality and specificity.

Discussion

RA displays pronounced anticarcinogenic activities in several types of cancer, but the exact mechanisms that underlie these activities remain incompletely understood. Previous reports (11, 26, 27) and the data presented here indicate that RA-induced growth inhibition of mammary carcinoma MCF-7 cells comprises a two-phase process, entailing an early cell cycle arrest followed by induction of apoptosis. The present work aimed to obtain insights into the initial cell cycle response. We show that 1 to 3 days of RA treatment results in a decrease in cyclin D1 levels and in a cell cycle arrest in the G_1 phase (Fig. 1). The data further show that

[1] http://www.gene-regulation.com.

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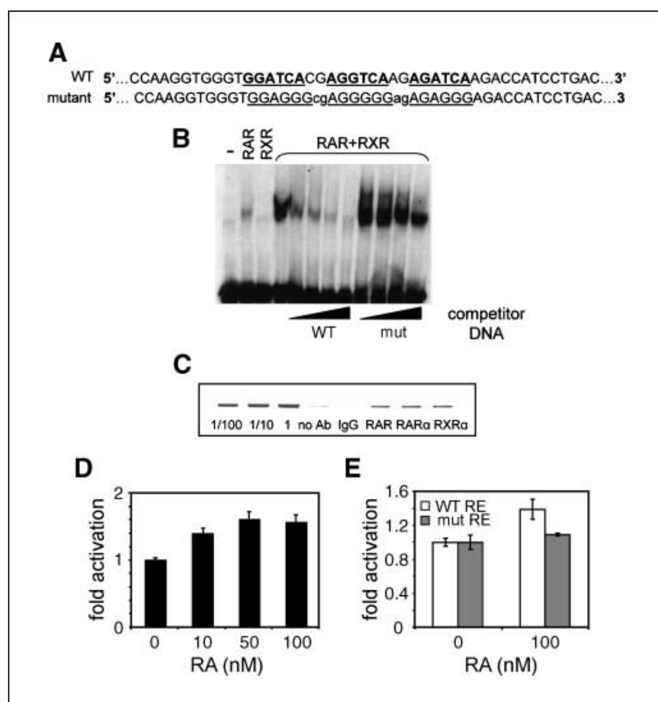

Figure 5. A functional RARE in the Btg2 promoter. *A,* sequence of the *Btg2* promoter at −3,357 to −3,142 bp. Putative RAREs in the wild-type promoter (*WT*) are boldfaced and underlined. Mutated elements in mutant oligonucleotides are underlined. *B,* electrophoretic mobility shift assays were carried out using bacterially expressed RARαΔAB (100 nmol/L), RXRαΔAB (100 nmol/L), and an oligonucleotide containing the putative RARE (*A*, 1 ng). Competition assays used increasing amounts of cold wild-type or mutated (*mut*) oligonucleotide (100, 200, 400, and 800 ng). *C,* chromatin immunoprecipitation assay of the *Btg2* promoter. PCR was done on materials immunoprecipitated with pan-RAR (RAR), RARα, or RXRα antibodies to amplify the RARE-containing region of the *Btg2* promoter (−3447 to −3197). *D* and *E,* MCF-7 cells were transfected with a luciferase reporter construct driven by the *Btg2* RARE (*D*) or the wild-type and mutant RARE construct (*E*), and transactivation assays were carried out as described under Materials and Methods. *Columns,* mean (*n* = 3); *bars,* SD.

a short-term RA treatment of MCF-7 cells is sufficient for triggering the program that leads to growth inhibition several days later (Fig. 3). These observations thus suggest that growth inhibition by RA is mediated by induction of immediate RAR target genes, and that the effect does not require the presence of the hormone throughout the progression of the program. Notably, although a RA pulse efficiently induced cell cycle arrest, it did not trigger the apoptotic response observed in cells that were continuously treated with RA for 4 days (Fig. 3).

It has been reported that the half-life for degradation of RA in F9 teratocarcinoma cells and in the kidney cell line LLC-PK1 is 3.5 and 2.3 h, respectively (34, 35). In MCF-7 cells, RA degradation seems to be comprised of a two-phase process, displaying half-lives of 13 and 84 min, respectively (Fig. 2; mean of two experiments). These rates are faster than those reported for other cell lines, but it should be noted that in MCF-7 cells, expression of CYP26a, an enzyme that specifically catalyzes RA metabolism (36), increases by ∼18-fold following a 4-h RA treatment (11). The rapid rates of RA degradation observed here may thus reflect the dramatic up-regulation of CYP26 resulting from the pretreatment of the cells with RA.

The two-phase process by which RA is degraded in MCF-7 cells may reflect the existence of two parallel degradation processes: a rapid process characterized by a low K_m and a high V_{max} and a second process displaying a high K_m and a low V_{max}. Alternatively, the behavior may reflect the existence of two intracellular RA pools; for example, free and protein-bound RA may degrade at different rates. Such a behavior has been reported for a number of substrates as exemplified by the observations that free retinol can be esterified by acyl-CoA/retinol acyltransferase and by lecithin/retinol acyltransferase (LRAT), whereas retinol bound to cellular retinol binding protein is recognized solely by LRAT (37–39). Our observations that reducing the expression level of CRABP-II in MCF-7 cells did not affect the rate of degradation of RA suggest that this binding protein is not involved in regulating the catabolism of its ligand, but it is possible that the CRABP-II homologue CRABP-I, which is also expressed in MCF-7 cells (10), serves in such a capacity.

The expression of several genes involved in cell cycle regulation was found to be up-regulated following a 4-h treatment of MCF-7 cells with RA (Table 1). Of particular interest are the observations that RA induces the expression of *Btg2*, a member of the antiproliferative (APRO) family. Btg2, which was originally identified as an immediate-early gene induced by nerve growth factor in PC12 cells (40, 41), is involved in regulation of cell growth and differentiation. It was thus reported that *Btg2* is a direct target for the tumor suppressor p53 (42–44); that it is induced in response to DNA damage in a p53-dependent manner (44, 45); that it triggers cell cycle arrest by inhibiting the G_1-S transition; and that this effect is exerted by decreasing the expression of cyclin D1 (30, 46, 47). Recent observations showed that, similarly to its activities in PC12 and NIH3T3 cells, overexpression of Btg2 in MCF-7 cells down-regulates the expression of cyclin D1 and inhibits cell growth (48). It was also recently shown that Btg2 plays a key role in mediating the ability of p53 to suppress Ras-induced transformation of mouse and human fibroblasts (43). In accordance with an antiproliferative function, the expression of Btg2 is reduced or lost in various carcinoma cells and human cancers, including prostate, breast, kidney, and stomach cancers (31, 32, 43, 46). Although the mechanisms by which Btg2 exerts its antiproliferative activities are incompletely understood, it has been suggested that it modulates transcriptional activities, and that it does so by interacting with transcriptional coactivators or corepressors. It was thus reported that Btg2 enhances the activity of protein-arginine methyltransferase 1; that it associates with homeobox b9 and carbon carbonylate repressor 4–associated factor 1; and that it may interact with the estrogen receptor α (49–52).

We show here that *Btg2* comprises a direct target for RA-induced, RAR-mediated, transcriptional signaling. In support of this conclusion, the data show that RA induces the expression of *Btg2*, and that the induction does not require *de novo* protein synthesis (Fig. 4). Additional observations show that an element containing three RARE half-sites is present 3,250 bp upstream of the *Btg2* start site, and that this element specifically binds RXR-RAR heterodimers *in vitro*, is occupied by the heterodimers in MCF-7 cells, and can drive RA-induced transcriptional activation of a luciferase reporter (Fig. 5). In view of the previous report that Btg2 induces cell cycle arrest by down-regulating the expression of cyclin D1 (23), the observations that RA treatment markedly decreased cyclin D1 expression (Fig. 1*B*), further suggest that Btg2 plays an important role in mediating the antiproliferative activities of RA in MCF-7 cells. Taken together, the observations show that RA induces a G_0-G_1 arrest in MCF-7 cells by up-regulating the expression of several genes involved in cell cycle control, and that a short-term treatment with RA is sufficient to

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elicit the response. Hence, the antiproliferative activity of RA in these cells is mediated, at least in part, by induction of Btg2 expression.

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