The Estrogen-responsive B Box Protein Is a Novel Regulator of the Retinoid Signal*

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Retinoic acid (RA) induces growth arrest, cell death, and differentiation in many human cancer cells in vitro and has entered routine clinical use for the treatment of several human cancer types. One mechanism by which cancer cells evade retinoid-induced effects is through repression of retinoic acid receptor β (RARβ) gene transcription. The RA response element β (RARE) is the essential DNA sequence required for retinoid-induced RARβ transcription. Here we show that the estrogen-responsive B box protein (EBBP), a member of the RING-B box-coiled-coil protein family, is a βRARE-binding protein. EBBP undergoes serine threonine phosphorylation and enhanced protein stability after RA treatment. Following RA treatment, we also observed increased nuclear EBBP levels in aggregates with the promyelocytic leukemia protein at promyelocytic leukemia nuclear bodies. EBBP enhanced RA-responsive RARβ transcription in RA-sensitive and -resistant cancer cells, which were resistant to both a histone deacetylase inhibitor and a demethylating agent. EBBP-specific small interfering RNA reduced basal and RA-induced RARβ expression. EBBP increased βRARE-transactivating function through its coiled-coil domain. Taken together, our work suggests that EBBP may have a pivotal role in the retinoid anti-cancer signal.

Over the past decade, basic and clinical research has demonstrated a therapeutic role for retinoids, in particular RA2 in human cancer (1–3). Molecular events at the point of transcriptional regulation appear to be critical in determining cellular responses to retinoids. Upon entering the nucleus by simple diffusion, the retinoid ligand binds to retinoic acid receptors (RARs) or retinoid X receptors (each with α, β, and γ subtypes). The ligand-receptor complex initiates the retinoid signal by changes that occur at consensus DNA sequences or RA response elements (RAREs) in the regulatory 5’-untranslated sequence of so-called “target” genes.

RARβ is a retinoid target with a well defined βRARE, which is necessary but not sufficient for the RA anti-cancer signal (4). Repression of basal or RA-induced RARβ transcription is a common event in a wide range of human tumor types, and numerous studies indicate that in specific circumstances RARβ acts as a tumor suppressor gene (2, 5–8). A frequent mechanism of RARβ repression is DNA methylation-induced silencing (9). Additionally, histone deacetylation is also associated with retinoid resistance, in the presence and absence of RARβ2 hypermethylation, indicating that multiple mechanisms appear to contribute to RARβ repression (10).

Intrinsic and acquired retinoid resistance has limited the clinical activity of retinoid-based therapy and chemoprevention. RA treatment in acute promyelocytic leukemia has been particularly valuable in illuminating mechanisms of retinoid resistance. Most acute promyelocytic leukemia cases present with the reciprocal t(15,17) translocation, resulting in the fusion product PML-RARa (11). Dominant negative effects of the PML-RARa fusion protein have been associated with increased affinity for co-repressor proteins in acute promyelocytic leukemia cells. The signaling is constitutively repressed by co-repressor-associated histone deacetylase activity at physiologic RA levels. Co-repressor association with PML-RARa is disrupted, following recruitment of co-activator complexes and differentiation induced at pharmacologic RA dosages (12–14). PML protein localizes to discrete nuclear domains termed PML bodies, which represent one of many subnuclear domains in the eukaryotic cell nucleus (15, 16). Treatment of acute promyelocytic leukemia cells in culture with RA correlates with degradation of the PML-RARa fusion protein, allowing wild-type PML protein to dimerize or multimerize in PML bodies (17).

In common with other hormone receptor proteins, RARs can act as either transcriptional repressors or activators (18). This dual mode of regulation is dependent on the ability of RARs to associate with either co-activator or co-repressor proteins. Retinoid ligand binding to nuclear retinoid receptors induces a conformational change in these proteins, already bound at target gene promoter sequences, leading to new protein-protein interactions. One of the key factors determining the transcriptional and cellular response to RA is the dynamic change in co-regulator protein binding. These interactions are determined by the nuclear location, the post-translational modification, and the expression level of co-regulator proteins (19–21).

Acetylation, methylation, or phosphorylations of specific residues in histone tails, mediated by multiprotein complexes, control the activity of nuclear receptors. Most nuclear hormone receptors are phosphoproteins, and phosphorylation is a potent regulator of nuclear receptor
function. Depending on the receptor, phosphorylation can regulate receptor DNA binding, dimerization, coactivator recruitment, and transactivation (22–26). These findings led us to hypothesize that the retinoid anti-cancer effect may be determined by other proteins that undergo post-translational modification following retinoid treatment of cancer cells, and, moreover, that such changes in co-regulator proteins may represent mechanisms for overcoming retinoid resistance in cancer cells.

To identify novel regulators of retinoid signaling, we developed a technique for directly isolating RARE-binding proteins, using electrophoresis, two-dimensional polyacrylamide gel electrophoresis (2-DE), direct isolation of differentially displayed protein spots, and peptide microsequencing to better understand the dynamic changes in protein-protein and protein-DNA complexes at this focal point of the RA anti-cancer signal. Using this approach, we have identified the estrogen-responsive B box protein (EBBP) as a RARE-binding protein, which undergoes post-translational modification, affecting protein stability and expression level at PML bodies, following RA treatment of cancer cells. Importantly, EBBP depressed RA-responsive RARβ transcription in retinoid-resistant cancer cells, suggesting a novel mechanism for RA-regulated gene transcription.

MATERIALS AND METHODS

Cells and Culture Conditions—The human neuroblastoma cell lines used in these experiments were BE(2)-C (BE) and SH-SY5Y (SY). BE and SY cell lines were generously supplied by Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York). The human lung cancer cell line (SK-MES-1) and breast cancer cells (MDA-MB-231 and MDA-MB-468) were obtained from the American Type Culture Collection. All cells were cultured at 37°C in 5% CO2 as an adherent monolayer in Dulbecco’s modified Eagle’s medium supplemented with 1-glutamine and 10% fetal calf serum. All-trans-retinoic acid (aRA) was purchased from Sigma, solubilized in ethanol, stored at −70°C, and used within 2 weeks of solubilization. 5-Aza-2-deoxycytidine and trichostatin A were purchased from Sigma.

Decoy Experiments—RARE decoy (GAT CGG GTA GGG TTC ACC GAA AGT TCA CTC G) or scrambled (AGC TCT GTG TTC TCC TGG TGT TCT GAA GCT) oligonucleotides were annealed to their respective complementary sequence by heating to 80°C for 5 min and then cooling down to room temperature. BE cells cultured in T25 flasks were transfected with either RARE-binding proteins, using electrophoretic mobility shift assay, the polyacrylamide gels were transferred onto 3 MM blotting paper (Whatman) and dried on a slab gel drier prior to autoradiography. Protein-RARE complexes were excised directly from the gels and then passively eluted from the DNA-protein complexes (passive elution buffer: 100 mM sodium acetate, 0.1% SDS, and 10 mM dithiothreitol). The proteins were then separated using 2-DE, which was performed according to the manufacturer’s instructions. The cells were co-transfected with 2 μg of the pGL3-3×RARE luciferase (firefly luciferase) reporter plasmid, 2 μg of EBBP expression vector, and 0.75 μg of the pRL-TK vector (Renilla luciferase) as an internal control for the transfection efficiency. After 48 h, the treated cells were washed with phosphate-buffered saline (PBS), harvested by scraping in 250 μl of passive lysis buffer (Promega), and lysed by freezing/thawing two times. Lysate samples were cleared by centrifugation for 30 s at 4°C. The supernatants were used for measuring both firefly and Renilla luciferase activities with the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (TD-20/20; Turner Designs, CA). Luciferase reagent substrate (100 μl) was mixed with cell lysates (20 μl), and then initiated the Renilla luciferase reaction. Stop & Glo solution (100 μl) was added to the mixture, and Renilla luciferase activity was measured. The ratio between firefly and Renilla luciferase activities was referred to as relative luciferase activity.

Analysis of Gene Expression by RT-PCR—RARβ and EBBP mRNA expression levels in neuroblasts were measured by competitive semiquantitative RT-PCR technique, which we have previously described (30). The RT-PCR primer involved determining a ratio between the level of expression of the RARβ and EBBP genes and that of the control β2-microglobulin (β2M) gene in total RNA samples. The RT-PCR primer sequences for β2M have been described (31). The gene-specific RARβ forward PCR primer was 5′-CTCACGTGCCAGTCCGGCTCTT-3′, and the reverse PCR primer was 5′-CAGAGCTGTTGCTCTGTGTT-3′. This primer pair generated a 131-bp product extending from bp 275 to 506 of the published RARβ sequence (4). The gene-specific EBBP forward PCR primer was 5′-CAGAGCTCCAGCTTACTACAAAG-3′, and reverse PCR primer was 5′-TCCCTTAA- GAAGGGCATACATTG-3′. This primer pair generated a 131-bp PCR product extending from bp 812 to 943 of the published EBBP coding sequence (32). In order to maximize RT-PCR efficiency in RA-resistant SK-MES-1 cells, the FailSafe PCR System (Epicerin Biotech-
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tologies) was used. This involved using the FailSafe PCR enzyme mix and a specific PCR C buffer.

Quantitative Real Time PCR—RARβ mRNA expression levels in lung and breast cancer cells were assayed using a quantitative real time PCR technique. Total RNA was isolated from cell lines using RNeasy Mini Kit (Qiagen). For each RT-PCR, 2 μg of total RNA was converted into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega). Two microliters of reverse transcription reaction was then quantified by quantitative real time PCR using PRISM 7700 Sequence Detection System and ABI TaqMan® PCR Master Mix (Applied Biosystems). The quantitative results of RARβ mRNA were normalized to β2M mRNA from the same sample. Primers were synthesized at Invitrogen, and probe was purchased from Applied Biosystems. Primers used for RARβ were as follows: forward primer, 5′-tgaaatcacagatctccgtgac-3′ and reverse primer, 5′-ccaggagtttcacctttcaagtt-3′. The TaqMan® probe used for RARβ was as follows: 5′-6-carboxyfluorescein-cctgcacctttagcactg-3′ molecular groove-binding non-fluorescence quencher. Primers used for β2M were as follows: forward primer, 5′-actgtctattctgctgtaagctc-3′; reverse primer, 5′-tgatgctgcttacatgtctcgat-3′. The TaqMan® probe used for β2M was 5′-VIC-tgcctgcacctttagcactg-3′ 6-carboxy-tetramethyl-rhodamin.

Immunofluorescence Staining for EBBP and Myc Tag Antibodies—Immunohistochemical staining for EBBP expression was carried out as follows. All procedures were performed at room temperature. BE cell cultures adherent to the substratum on the glass slide were rinsed in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) in wells. Cells were fixed and permeabilized by incubation in 500 μl of cold 4% paraformaldehyde in TBS for 20 min prior to incubation in 500 μl of 0.2% Triton X in TBS in wells for 10 min. After washing in TBS, cultures were incubated in 100 μl of blocking buffer 1 (1% fetal bovine serum, 1% bovine serum albumin in TBS) and then incubated in 100 μl of polyclonal rabbit anti-human EBBP antibody diluted 1:30 in TBS or monosclonal mouse anti-Myc tag antibody diluted 1:500 in TBS in wells for 10 min. After washing in TBS, antibodies were incubated in 10 μl of blocking buffer 2 (1% fetal bovine serum, 1% bovine serum albumin in TBS) and then incubated in 100 μl of primary antibody (anti-EBBP or anti-Myc tag) diluted 1:100, 1:500, 1:1000, 1:5000 in 5% fetal calf serum, PBS for 1 h. After washes in TBS, cells were incubated in 100 μl of secondary antisera, goat anti-rabbit fluorescein isothiocyanate diluted 1:150 in TBS for 1 h or Cy3 anti-mouse secondary antibody diluted 1:1000 in 5% fetal calf serum, PBS buffer for 40 min. After washing, slides were mounted in fluorescence anti-fade solution and sealed with clear nail polish.

Confocal Scanning Fluorescence Microscopy Images of EBBP and PML Body Immunostaining—Cells were fixed in 10% formalin for 20 min, followed by permeabilization with 0.2% Triton X for 10 min. Cells were then blocked in 1% fetal bovine serum and 1% bovine serum albumin in PBS for 1 h and then incubated in rabbit anti-EBBP (1:30) for 1 h, followed by anti-rabbit fluorescein isothiocyanate (1:100) for 1 h. Cells were then reblocked for 10 min in 10% bovine serum albumin in PBS before incubation in mouse anti-PML (1:10) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h and then incubated in biotinylated anti-mouse IgG (1:100) for 1 h and finally labeled with streptavidin Alexa 594. Coverslips were then mounted on slides in Vectashield antifade solution and sealed with nail polish ready for fluorescence microscopy.

Confocal scanning fluorescence microscopy was performed on a TCS SP2 System (Leica) fitted with a HCX PL APO ×63/1.20 W CORR objective. Fluorochromes fluorescein isothiocyanate and Alexa 594 were excited with the 488-nm argon laser and the 530-nm helium/neon laser, respectively. Emission fluorescence was collected in red (>580 nm) and green (500–560 nm) channels sequentially. Images were collected and processed using the imaging software provided by the Leica TCS NT system. Images were randomly taken from at least three different fields of view, and the immunofluorescence was measured from 6–8 cells/treatment. Nuclear areas were considered EBBP-positive if they displayed a fluorescence of over 1501 (Arbitrary Fluorescence Unit).

Immunoblot Analysis and Protein Degradation Determination—Immunoblot analysis and 2-DE immunoblot of nuclear proteins (50–150 μg of protein/lane, respectively) were performed as previously described (6). Crude EBBP antisera was kindly provided by Dr. D. A. Zachowics (Berlex Biosciences). A synthetic peptide (TNTTPWE-PPDDLPS), representing the EBBP protein sequence residues 396–410, was conjugated to keyhole limpet hemocyanin. Polyclonal antibody was generated by immunizing rabbits with the conjugated peptide following a standard immunization protocol (32). Mouse monoclonal antibody anti-Myc tag (9B11) was purchased from Cell Signaling Technology Inc. The EBBP antibody for immunoblot and 2-DE immunoblot was used at a 1:250 dilution, and anti-Myc tag antibody for EBBP deletion mutant immunoblot was used at a 1:500 dilution in 0.5% defatted milk in TBST buffer. Rabbit polyclonal actin antibody (Sigma) was used at a 1:2000 dilution to normalize for differences in loading. Subsequently, membranes were incubated with horseradish peroxidase, anti-rabbit, or anti-mouse secondary antibody (Pierce), which was used at a 1:5,000 dilution. Chemiluminescent detection was performed using the Super-Signal Western blotting Kit (Pierce) according to the manufacturer’s instructions.

To determine the EBBP protein stability, BE cells were treated with or without cycloheximide (3.35 μM) and aRA (10 μM). At the indicated time points, cell cultures were harvested, and nuclear extracts were prepared using an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce). Equal amounts of protein (50 μg) from each sample were analyzed by Western blotting using an anti-EBBP or anti-RARβ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:400 dilution. The relative amounts of EBBP and RARβ proteins were quantified using a Bio-Rad Quantity One program. Identical blots were analyzed by Western analysis using anti-actin antibodies as the loading control.

Bio-Plex Phosphoprotein Assays—For analysis of phosphoproteins, neuroblastoma cells were treated with 10 μM aRA for 0, 3, 6, 9, and 24 h on 24-well plates. The cell lysates were prepared using the Bio-Plex cell lysis kit (Bio-Rad) according to the manufacturer’s instructions. Protein recovery was determined with a bicinchoninic acid protein assay.
EBBP is a candidate RARE-binding protein during aRA treatment. A, 2-DE analysis of candidate RARE-binding proteins. Purified proteins were analyzed by 2-DE over immobilized pH gradient (IPG) strips of pH 3–10, transferred to 4–15% Tris-HCl gradient Ready Gels, and visualized by SYPRO Ruby staining. The spots that were considered differentially expressed from each gel were excised, trypsin-digested, and analyzed by MALDI-MS. B, schematic representation of IPG strips of pH 3–10, transferred to 4–15% Tris-HCl gradient Ready Gels, and visualized by SYPRO Ruby staining. The spots that were considered differentially expressed from each gel were excised, trypsin-digested, and analyzed by MALDI-MS.

Measurement of DNA Synthesis—Incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) was determined to evaluate DNA synthesis by a proliferation kit (Roche Applied Sciences) according to the instructions provided by the manufacturer. The cells were seeded at 6 \times 10^5 cells/well in 96-well plates for 24 h and transfected with expression vectors for 72 h. The cells were incubated with BrdUrd (10 \mu M) for another 2 h and fixed, and the incorporated BrdUrd was labeled with a peroxidase-conjugated anti-BrdUrd antibody before the addition of a peroxidase substrate. The absorbance of the wells was measured on a microplate reader with a test wavelength of 370 nm and a reference wavelength of 490 nm.

Small Interfering RNA (siRNA)—siRNA for EBBP (ID 3573) and negative control siRNA (catalogue number) were purchased directly from Ambion as double-stranded EBBP predesigned siRNA. The sequences of the siRNA were targeted EBBP exon 6; the sequences were patented by Ambion as double-stranded EBBP predesigned siRNA. The sequences provided by the manufacturer were used in the siRNA experiment without modification.
the manufacturer’s instructions with minor modifications. EBBP-specific antibody and control rabbit IgG antibody (Santa Cruz Biotechnology) were used in the experiments. Chromatin was immunoprecipitated from \(2 \times 10^6\) BE cells transiently transfected with empty vector or pcDNA3.1(−)/EBBP construct. For PCR, the primers included the RARP2 sense primer \(5'-\)GCCGAGAAGCCTGACCTG-3' and the RARP2 antisense primer \(5'-\)GGCCCAATCCAGGCCCGGC-3' (6).

**RESULTS**

Isolation of Candidate βRARE-binding Proteins during RA-induced RARβ Transcription—To provide evidence that the specific RARE in the RARβ2 gene promoter was required for RA-induced RARβ induction, we transiently transfected RA-sensitive human neuroblastoma BE cells with a double-stranded decoy oligonucleotide that corresponded to the βRARE (4) to compete for and block RA-activated receptor complex binding at the βRARE. Transfection of the βRARE decoy oligonucleotide, but not the scrambled oligonucleotide, significantly inhibited RA-induced RARβ mRNA expression (Fig. 1). We next performed an electrophoretic mobility shift assay with the βRARE oligonucleotide and nuclear protein lysates from aRA-treated and -untreated BE cells at different time points following aRA treatment. Protein-DNA complexes were directly isolated from the polyacrylamide gel and then electrophoretically separated on 2-DE. Protein gels were compared for differential protein display over the 48 h of aRA treatment. Protein spots were excised directly from the gel, and microsequencing of tryptic peptides was determined by MALDI-MS analysis. Using this technique, we identified EBBP as a candidate βRARE-associated protein of 67 kDa, which exhibited an alteration in pH from pH 5.4 to 6.0 during aRA treatment, suggesting post-translational modification (Fig. 2A). The peptide masses were searched against Swiss-Prot using PeptIdent (available on the World Wide Web at www.expasy.ch/tools/peptident.html) (Fig. 2C). To confirm these findings, we transiently transfected BE cells with
EBBP was first identified in a screen for transcripts induced by estrogen in human mammary epithelial cells (32) and is a member of the RBCC (34) or tripartite motif (TRIM) (35) protein family, sharing the B box and coiled-coil domains with PML and transcriptional intermediary factor (TIF1α) (Fig. 2D). When compared with PML and TIF1α, EBBP is missing the NH₂-terminal RING finger domain but has an additional COOH-terminal RFP or B30.2 domain of unknown function. This additional domain is shared with several other TRIM proteins, such as MID1 and PYrin, which have been shown to be mutated in the human disorders Opitz syndrome and familial Mediterranean fever, respectively (36). Next we used the ChIP assay with EBBP and acetylated histone H4 antibodies to analyze in vivo the composition of proteins binding to the βRARE of the RARβ2 promoter in EBBP transfectants (Fig. 2E). Overexpression of EBBP caused EBBP binding and acetylation of histone H4 at the βRARE of the RARβ promoter when compared with empty vector transfectants.

**RA Alters EBBP Protein Stability**—In order to examine the mechanism of the aRA-induced increase in EBBP expression, we measured the level of EBBP mRNA expression in RA-responsive BE and SY neuroblastoma cells, treated with 10 μM aRA for 0 h, 1 h, 24 h, 3 days, and 7 days, using gene-specific PCR primers and control β2M primers in a semiquantitative RT-PCR. These cell lines undergo significant growth arrest, cell death, and neuritic differentiation following aRA treatment (37). RT-PCR analysis showed no change in EBBP mRNA level during aRA treatment (data not shown). However, EBBP protein levels increased in aRA-treated cells by more than 2-fold in immunoblotting experiments using an EBBP-specific antibody in both cell lines (Fig. 3A). To determine if aRA treatment affected EBBP protein stability, BE cells were treated with the protein synthesis inhibitor, cycloheximide, in conjunction with retinoid (Fig. 3B). The aRA-induced levels of EBBP protein expression increased in a similar manner in both the cycloheximide-treated and -untreated cells over 24 h, in contrast to aRA-induced expression of RARβ, which required new protein synthesis (data not shown). Nuclear extracts from cells treated with cycloheximide only (absence of RA) were subjected to Western analysis, which showed no changes in EBBP protein level during the time course (Fig. 3C).

**Post-translation Modification of EBBP**—To investigate the nature of the RA-induced post-translational modification of EBBP, we examined the phosphorylation status of EBBP protein during 24 h of RA treatment. Protein lysates from BE cells treated with aRA were incubated with polystyrene beads that had been coupled to an EBBP-specific antibody before being analyzed with a dual laser, flow-based microplate reader system (Bio-Plex suspension array system). This system identifies both antibodies and shows the level of phosphorylated EBBP protein in the well as fluorescence intensity using Bio-Plex Manager software (Fig. 3D). In contrast to untreated cells, EBBP protein phosphorylation levels increased 5-fold at 6 and 9 h following aRA treatment, whereas EBBP phosphorytosine levels were relatively unchanged.

**Retinoi-induced EBBP Protein Co-localizes with PML to Nuclear Bodies**—We next determined the pattern of subcellular localization of EBBP following RA treatment, using confocal scanning fluorescence microscopy. EBBP was first identified in a screen for transcripts induced by estrogen in human mammary epithelial cells (32) and is a member of the RBCC (34) or tripartite motif (TRIM) (35) protein family, sharing the B box and coiled-coil domains with PML and transcriptional intermediary factor (TIF1α) (Fig. 2D). When compared with PML and TIF1α, EBBP is missing the NH₂-terminal RING finger domain but has an additional COOH-terminal RFP or B30.2 domain of unknown function. This additional domain is shared with several other TRIM proteins, such as MID1 and PYrin, which have been shown to be mutated in the human disorders Opitz syndrome and familial Mediterranean fever, respectively (36). Next we used the ChIP assay with EBBP and acetylated histone H4 antibodies to analyze in vivo the composition of proteins binding to the βRARE of the RARβ2 promoter in EBBP transfectants (Fig. 2E). Overexpression of EBBP caused EBBP binding and acetylation of histone H4 at the βRARE of the RARβ promoter when compared with empty vector transfectants.

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microscopy in BE cells (Fig. 4). In untreated BE cells, EBBP staining was scattered throughout the nucleus. Following 10 μM aRA treatment, however, the nuclei of aRA-treated cells displayed an increase in the intensity of EBBP staining at 24 h, which had coalesced into a single, strongly stained region by 48 h (Fig. 4A). Quantitative analysis of EBBP-positive regions within the nuclei of untreated or aRA-treated cells demonstrated that the number of EBBP-positive regions increased within 2 h of RA treatment and peaked at 24 h before declining at 48 h (Fig. 4B). The strongly staining nuclear EBBP region seen at 48 h after aRA treatment resembled the large multiprotein complexes, known as PML nuclear bodies (15, 16). Accordingly, we examined whether EBBP and PML proteins co-localized to PML bodies. PML, like EBBP, had a scattered, nuclear distribution and was expressed in every cell. PML expression did not discernibly change in level or localization with aRA treatment (data not shown). Under confocal fluorescent microscopy, PML co-localized with EBBP in 50% of untreated or aRA-treated cells. However, at 48 h of aRA treatment, EBBP appeared to aggregate into large nuclear bodies and was co-localized with PML (Fig. 4C).

Overexpressed EBBP Induces Growth Inhibition and Enhances RARβ Transcription and Transactivation through the βRARE in Retinoid-sensitive Neuroblastoma Cells—To further define the role of the EBBP protein in determining retinoid sensitivity of neuroblastoma cells, we transiently transfected human EBBP cDNA into the cells. Transient overexpression of EBBP led to a significant decrease in viable BE cells numbers when compared with empty vector control (p < 0.05), as measured by trypan blue exclusion at 72 h after transfection. In the cell proliferation study, BrdUrd incorporation by BE cells was decreased by 50% in cells transfected with EBBP plasmid DNA, after 48 h of aRA treatment or without treatment, compared with empty vector control (p < 0.01) (Fig. 5A). The functional consequences of RA-induced EBBP expression were assessed by examining the effect of increased EBBP expression on endogenous RARβ transcription and transactivation from the βRARE. We transiently transfected full-length human EBBP cDNA and empty vector into retinoid-sensitive BE and SY neuroblastoma cells. Transfection of EBBP increased endogenous RARβ mRNA expression following 10 μM aRA treatment in both neuroblastoma cell lines at 24 and 48 h of aRA treatment compared with empty vector control (Fig. 5B). We next evaluated whether an excess of EBBP would enhance the transactivating ability of a transiently transfected βRARE-luciferase construct in the presence of aRA. Following 24 and 48 h of aRA treatment, both cell lines demonstrated a 1.5–2.0-fold increase in βRARE-luciferase activity, when EBBP was co-transfected with βRARE-luciferase, in comparison with the empty vector alone (Fig. 5C), suggesting that RA-induced EBBP expression has functional consequences for endogenous RARβ gene expression.

Overexpressed EBBP Enhances RARβ Transcription and Reduces Cell Proliferation in Retinoid-resistant Lung and Breast Cancer Cells—The effects of an excess of EBBP were examined in retinoid-resistant SK-
MES-1, MDA-MB-231, and MDA-MB-468 cancer cells, which are known to be phenotypically resistant to aRA and do not induce RARβ/H9252 transcription following aRA treatment (38). To determine whether the repression of RARβ/H9252 expression in the aRA-resistant cancer cells was due to DNA methylation, these cancer cells were treated with the demethylation agent, 5-aza-2-deoxycytidine. Treatment with 0.1, 1.0, and 10 μM 5-aza-2-deoxycytidine in combination with 10 μM aRA for 3 days did not restore RARβ/H9252 expression in the aRA-resistant cells when analyzed by RT-PCR (data not shown). Since the level of histone acetylation can influence gene transcription, the effects of the histone deacetylase inhibitor, trichostatin A, on RARβ/H9252 expression in SK-MES-1, MDA-MB-231, and MDA-MB-468 were investigated. Cells were treated with 0.3 μM trichostatin A (39) in combination with 1 μM aRA for 24 h and 3 days and analyzed for RARβ/H9252 expression. RARβ transcription was not detected in these cancer cells by RT-PCR (data not shown). However, when SKMES-1 MDA-MB-231 and MDA-MB-468 cells were transfected with an EBBP expression vector, endogenous RARβ mRNA expression at 24 and 48 h of aRA treatment was restored (Fig. 6A). Furthermore, proliferation of these cancer cells was also significantly reduced by transient overexpression of the EBBP expression vector with aRA treatment for 48 h compared with empty vector control measured by BrdUrd incorporation (Fig. 6B). These results suggested that overexpression of EBBP in retinoid-resistant cancer cells may overcome repression of retinoid-induced transcription, which was resistant to both demethylation and histone acetylation.

**Down-regulation of RARβ Gene Expression with Specific EBBP siRNA**—Next we examined the hypothesis that knockdown of EBBP expression will affect RA-responsive RARβ transcription. The efficiency of EBBP siRNA in reducing RARβ was evaluated after 72 h of transfection (Fig. 7). The siRNA concentration was kept as low as 10 nM in order to eliminate the possibility of nonspecific effects. A very efficient decrease in EBBP mRNA expression was obtained, with only 40% remaining expression in untreated BE cells and 18% remaining in 48-h 10 μM aRA-treated BE cells (Fig. 7A). RARβ mRNA expression was significantly decreased after transfection of 10 nM siRNA EBBP for 72 h in the presence of 10 μM aRA for 48 h and compared with control siRNA (p < 0.05) (Fig. 7B). As shown in Fig. 7C, the level of RARβ protein was decreased by 40% in the absence or presence of aRA treatment compared with control siRNA.

**The EBBP Coiled-coil Domain Is Necessary for the Effect of EBBP on βRARE Activity**—To determine which protein domain of EBBP was required for its effect on βRARE activity, we created four EBBP deletion mutant constructs, where the mutant protein was expressed with both a Myc epitope and a His6 tag (Fig. 8). We used both immunofluorescent staining and immunoblot with Myc epitope antibodies to confirm that the mutant proteins were expressed in BE cells following transient transfection (Fig. 8, A and B). Without aRA treatment, the βRARE-luciferase activity was increased in EBBP full-length, mutant 1, and mutant 3 constructs compared with empty vector control (p < 0.05). Following 48-h treatment with 10 μM aRA, only mutants 1 and 3, which each share the coiled-coil domain, retained the ability to enhance βRARE activity to the same degree as full-length EBBP (Fig. 8C).

**DISCUSSION**

EBBP is a poorly characterized member of the RBCC/TRIM family. In this report, we provide the first evidence that EBBP is a novel co-regu-
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FIGURE 7. Down-regulation of RARβ gene expression with specific EBBP siRNA. A and B, RT-PCR analysis of EBBP and RARβ mRNA expression at 72 h of BE cells without or with EBBP siRNA transfection as indicated in the figure. *, a statistically significant difference (p < 0.05) compared with negative control siRNA. **, a statistically significant difference (p < 0.01) compared with negative control siRNA. C, Western blotting analysis by RARβ-specific antibody at 72 h of BE cells without or with EBBP siRNA transfection. Actin was used as an internal control for loading.

lator of RARβ and, may play a significant role in the retinoid signaling pathway. Our data indicate that RA treatment of retinoid-sensitive cells results in EBBP phosphorylation and elevated EBBP nuclear expression in aggregates with PML at PML nuclear bodies. Overexpression of EBBP increases the level of exogenous and endogenous βRAR transactivation. Transfection of EBBP siRNA significantly decreased basal and RA-induced RARβ gene expression in neuroblastoma cells. A ChIP assay showed that EBBP protein directly interacted with the βRAR. Furthermore, overexpression of EBBP induced growth inhibition in RA-sensitive neuroblastoma cells as well as RA-resistant lung and breast cancer cells. Most importantly, EBBP restores RA-responsive RARβ transcription in RA-resistant lung cancer cells, which were resistant to both a histone deacetylase inhibitor and demethylating agent. In addition, deletion mutant analysis localized the βRAR transactivating function to the coiled-coil domain of EBBP, a region thought to be responsible for protein-protein interactions.

EBBP mRNA has previously been shown to be expressed in most tissues, whereas the protein appears to be present in a nascent and post-translationally modified form (32, 40). EBBP was independently identified recently in a screen for transcripts modified by the keratinocyte growth factor in keratinocytes (40). In these studies, keratinocyte growth factor caused early, transient down-regulation of EBBP in the first 24 h after treatment of keratinocytes in vitro. In vivo, EBBP was predominately expressed in undifferentiated keratinocytes in hyper-proliferative epithelium, and expression underwent a biphasic change during skin wound repair. Stable overexpression of EBBP in keratinocytes induced differentiation. Taken together, these findings suggested a role for EBBP in the response of tissues to differentiating agents and that changes in EBBP expression may be a dynamic component of the differentiation processes.

Our screen for changes in βRAR-associated proteins detected a 67-kDa protein whose isoelectric point (pI) changed from pH 5.4 to pH 6 during aRA treatment of RA-sensitive neuroblastoma cells. Since the calculated molecular mass is only 64 kDa, it seems likely that the protein is post-translationally modified and that this may vary dependent on the cellular context. Our results are consistent with previous data obtained with HepG2, COS-1, and HaCaT cells (32, 40). Phosphorylation is one form of post-translational modification that can produce a more acidic protein isoform on 2-DE SDS-polyacrylamide gel and so could not explain the shift to a more alkaline pH, which we observed in the initial 2-DE EBBP blot, following RA treatment. Our data indicate that, following RA treatment, EBBP undergoes serine-threonine phosphorylation and, in addition, an as yet unrecognized post-translational modification, which results in cathodal shift.

Cathodal shifts during 2-DE or isolectric focusing of proteins are typically observed when there is loss of sialic acid as a consequence of abnormal glycosylation (41, 42), loss of phosphate groups (43), or loss of negatively charged amino acids, such as aspartic or glutamic acid (44, 45). Loss of negatively charged residues with resultant heterogeneity in pl could result from alternative splicing (46) or post-translational proteolytic processing (47). EBBP has glutamic acid residues within close proximity to both the N and C termini, so it is conceivable that some proteolytic processing might alter its net charge and cause a cathodic shift. However, anomalous behavior during isolectric focusing of proteins has also been reported in the case of hemoglobin E7A and E6V/ E7A mutants (48). This kind of anomaly may also be due to the local effects of surrounding amino acids altering the theoretical pl or changes in protein-protein noncovalent interactions (49). Determination of the exact reason for the apparently anomalous behavior of EBBP in our case will require further experimental work.

Many transcription factors and their associated cofactors have been shown to oligomerize, and this property is often essential for their in vivo biological functions. Among the RBCC/TRIM family proteins, the PML1 and MDM1 proteins have been shown to participate in both hom- and hetero-oligomeric complexes (36, 50). Furthermore, another member of the RBCC/TRIM family, RFP, can hetero-oligomerize and co-localize with PML, and this interaction is necessary for the location of RFP nuclear bodies, which play an important role in regulation of cellular growth and differentiation (51). The apparent sequence and functional similarities between EBBP and PML suggested that EBBP might also undergo nuclear translocation, and indeed, this was the case. This suggests that EBBP may play an important role in the induction of the differentiation pathway in neuroblastoma cells.

In RA-sensitive neuroblastoma cells, we showed that EBBP enhanced RA-induced RARβ transcription, and this effect can be blocked by a specific EBBP siRNA that demonstrated that EBBP is an endogenous control point for RARβ gene expression. Most importantly, a similar result was also observed in RA-resistant lung (SK-MES-1) and breast (MDA-MB-231 and MDA-MB-468) cancer cell lines. We had previ-
over, that an excess of EBBP can overcome transcriptional repression in cells that are resistant to demethylation or histone acetylation, by mechanisms yet to be determined. Most importantly, overexpression of EBBP protein increased cell growth inhibition in several human cancer cells. Understanding the mechanism by which EBBP regulates cell cycle progression and defining an effective method to increase EBBP protein expression in human cancer cells may provide a novel strategy in cancer therapy.

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