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13. ABSTRACT (Maximum 200 Words)
Induction of apoptosis is a plausible approach for developing new cancer therapies. A class of synthetic retinoids related to 6-[3-(1-adamantlyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) potently induce apoptosis of both hormone-dependent and -independent breast cancers. We previously reported that apoptotic effects of AHPN/CD437 are mediated by mitochondrial targeting of orphan nuclear receptor TR3. In the past year, we have studied the mechanisms by which TR3 migrates from the nucleus to the cytoplasm and by which TR3 targets mitochondria. Our results demonstrate that the migration of TR3 from the nucleus to the cytoplasm requires retinoid X receptor (RXR) through their unique heterodimerization and is regulated by RXR ligands. We have also identified a nuclear export sequence in the RXR, which is required for its cytoplasmic localization. Furthermore, we have found that TR3 targets mitochondria through its interaction with Bcl-2 that resides at the outer mitochondrial membrane. The interaction converts Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule. Our results not only enhance our understanding of the molecular mechanism by which TR3 exerts its apoptotic effects in breast cancer cells but also provide novel approaches to induce apoptosis ob Bcl-2-expressing breast cancer cells by using RXR ligands.

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Table of Contents

INTRODUCTION

Although anti-estrogen tamoxifen shows activities in preventing development of breast cancer, there are no effective therapies for malignant estrogen-independent breast cancer, which is also often resistant to chemotherapy and γ-radiation therapy. Lack of such therapies has contributed to the high mortality for U.S. women. Novel therapies are therefore urgently needed.

Recent scientific progress has suggested that elimination of cancer cells through induction of cancer cell death (apoptosis) is a plausible approach for developing new cancer therapies. Retinoids, analogs of vitamin A, are promising chemopreventive agents for breast cancer. Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) were found to potently induce apoptosis of both hormone-dependent and –independent breast cancers (1), indicating that it may be representative of a novel class of compounds suitable for treatment of estrogen-independent breast cancers. We subsequently showed that TR3, an orphan member of the steroid/thyroid/retinoid receptor superfamily, is required for induction of apoptosis of lung and prostate cancer cells by AHPN/CD437 and other apoptotic stimuli (2). Moreover, we discovered that TR3, in response to apoptosis stimuli, is translocated from the nucleus to the cytoplasm, where it targets mitochondria to induce cytochrome c release and apoptosis (3, 4).

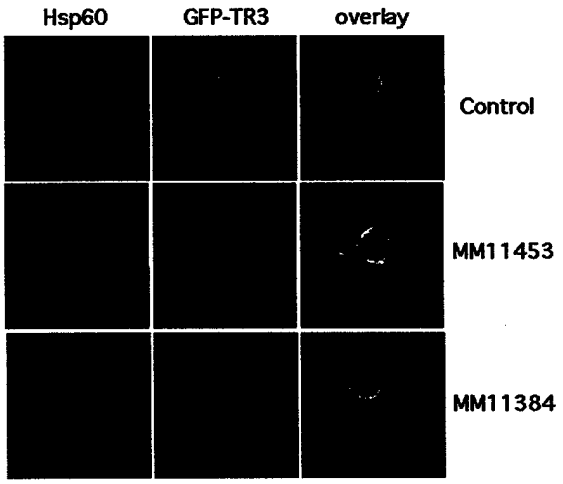
The objective of this application is to develop evidence that TR3 is a suitable molecular target for developing drugs against breast cancer. In the proposed studies, we will first establish whether TR3 is required for proliferation and apoptosis of breast cancer cells in response to various stimuli. We will then determine whether the novel nuclear-to-mitochondrial pathway for apoptosis occurs in breast cancer cells. In addition, we will study mechanism by which translocation of TR3 from the nucleus to cytoplasm is regulated by studying effect of phosphorylation. Furthermore, we will study physical interactions between TR3 and Bcl-2 and its effect on Bcl-2-mediated mitochondrial and post-mitochondrial activities. In the first year of research, we showed that retinoids AHPN analogs SR11453 and MM002 potently induced TR3 expression and apoptosis in breast cancer cells. Furthermore, we found that AHPN analogs induced migration of TR3 from the nucleus to mitochondria. We also observed that mitochondrial targeting of TR3 was regulated by ligands for retinoid X receptor (RXR) and that TR3 interacted with Bcl-2. In the past year, we have continued our studies on these directions and have made substantial progress as described below.

KEY RESEARCH ACCOMPLISHMENTS

1. Regulation of TR3 nuclear export by retinoid X receptor and its ligands.

Mitochondrial localization of TR3 in breast cancer cells. Fontana's group first showed that a new class of synthetic retinoids, related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) effectively induced apoptosis of both estrogen-dependent and -independent breast cancer cells (1), suggesting that these retinoids may represent a new class of drugs that have therapeutic value for the treatment of estrogen-independent breast cancer. We recently demonstrated that retinoid AHPN-induced apoptosis required expression of orphan receptor TR3, which migrated from the nucleus to mitochondria to trigger apoptosis (3). In our first year of research funded by BCRP, we showed that a green fluorescent protein (GFP)–TR3 fusion transfected into MDA-MB-231 cells migrated from the nucleus to the cytoplasm where it targeted to mitochondria when MDA-MB-231 cells were treated with two AHPN analogs, SR11453 and SR11384 (Figure 1A). Furthermore, we observed that GFP–TR3/ΔDBD (a mutant that lacks the DNA-binding domain) targeted mitochondria in MDA-MB-231 and ZR-75-1 breast cancer cells (Figure 1B).

A.



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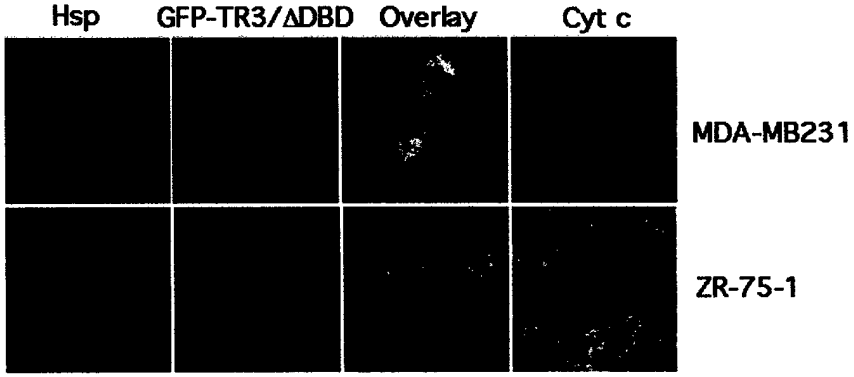

Figure 1. A. TR3 is targeted to mitochondria in MDA-MB-231 cells in response to an apoptosis inducer. GFP–TR3-transfected MDA-MB-231 cells were treated with or without SR11453 (10^{-6} M) or SR11384 (10^{-6} M) for 1 h, then immunostained with anti-Hsp60 antibody (Sigma), followed by Cy3-conjugated secondary antibody (Sigma) to detect mitochondria. GFP–TR3 and mitochondria (Hsp60) were visualized using confocal microscopy, and the two images were overlaid (see Overlay). B. **Mitochondrial targeting of TR3/ΔDBD is associated with cytochrome *c* release.** GFP–TR3/ΔDBD was transiently transfected into ZR-75-1 or MDA-MB-231 cells, which were stained for mitochondria (Hsp60) and cytochrome *c* (Cyt c), and analyzed by confocal microscopy.

RXRα targets mitochondria in response to apoptotic stimuli. How TR3 migrates from the nucleus to mitochondria to induce cytochrome c release is unknown. Since RXRα heterodimerizes with TR3 (5, 6), we studied whether RXRα targeted mitochondria in cells undergoing apoptosis. Subcellular localization of RXRα in LNCaP cells in the absence or presence of TPA, which potently induces LNCaP cell apoptosis, was examined by confocal microscopy analysis. Immunostaining showed that RXRα predominantly localized in the nucleus in the absence of TPA treatment (Figure 2A). However, when cells were treated with TPA, RXRα was found in the cytoplasm. To study whether RXRα was associated with mitochondria, cells were stained for heat shock protein 60 (Hsp60), a mitochondria-specific protein (Figure 2A). The extensive overlap in the distribution patterns of Hsp60 and RXRα suggested the association of RXRα with mitochondria. Similarly, treatment with TPA also resulted in mitochondrial localization of TR3 (Figure 2B), as previously reported (3).

RXRα and TR3 mitochondrial targeting are mutually dependent. To study whether TR3 and RXRα targeted mitochondria as a heterodimer, subcellular localization of TR3 and RXRα was examined in LNCaP cells treated with or without TPA. In the absence of TPA, both TR3 and RXRα resided mainly in the nucleus (Figure 2C). However, when cells were treated with TPA, TR3 and RXRα were colocalized in the cytoplasm and their distribution patterns overlaid extensively (Figure 2C), suggesting their association in the cytoplasm. Furthermore, transfected RXRα and TR3 associated and targeted mitochondria when cells were treated with an apoptosis inducer, the AHPN analog 3-Cl-AHPC (also called MM002) (7) that potently induced the apoptosis of cancer cells (Figure 2D). We next determined whether RXRα cytoplasmic localization depended on TR3 expression by examining its subcellular localization in LNCaP cells stably expressing TR3 antisense RNA (3). Expression of TR3 antisense RNA strongly inhibited TPA-induced TR3 expression in LNCaP cells. In contrast to that observed in wild-type LNCaP cells (Figure 2A), RXRα was found only in the nucleus in the TR3 antisense stable clone, even though the cells were treated with TPA (Figure 2E). To study whether TR3 mitochondrial targeting required RXRα, we used the siRNA approach to inhibit RXRα expression in LNCaP cells, then examined the subcellular localization of TR3. Transfection of LNCaP cells with RXRα siRNA strongly reduced RXRα protein levels (Figure 2F). In cells transfected with RXRα siRNA, TR3 was mainly confined in the nucleus despite TPA treatment (Figure 2F). Thus, cytoplasmic localization of TR3 and RXRα is mutually dependent.

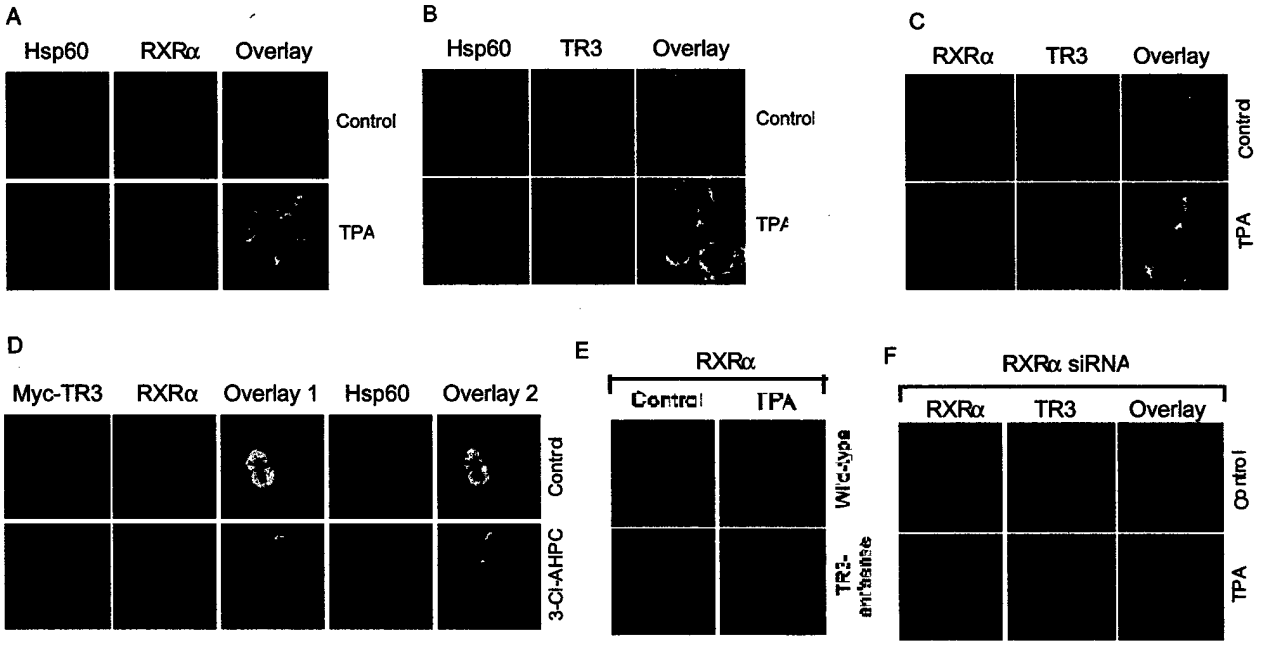

Figure 2. TR3 and RXR comigrate from the nucleus to the cytoplasm in LNCaP cells. RXRα (A) or TR3 (B) targets mitochondria in response to apoptotic stimulus. LNCaP cells were treated with TPA (100 ng/ml) for 1 hr, then immunostained with either anti-RXRα (Santa Cruz) (**A**) or anti-TR3 (**B**) antibody to detect RXRα or TR3 or with anti-Hsp60 antibody to detect mitochondria. RXRα, TR3 and mitochondria (Hsp60) were visualized using confocal microscopy, and the images of RXRα or TR3 were overlaid with those of mitochondria (overlay). **(C) TR3 and RXRα comigrate from the nucleus to the cytoplasm.** LNCaP cells were treated with or without TPA for 1 hr, then immunostained with anti-RXRα or anti-TR3 antibody. RXRα and TR3 were visualized using confocal microscopy and the images were overlaid (overlay). **(D) 3-Cl-AHPC induces mitochondrial localization of transfected RXRα and TR3.** Expression vectors for myc-TR3 and RXRα were transfected into LNCaP cells. Cells

were treated with 3-Cl-AHPC for 3 hr, then immunostained with anti-myc or anti-RXRα antibody. Myc-TR3, RXRα, and Hsp60 were visualized and the images were overlaid. In Overlay1, the myc-TR3 and RXRα images are overlaid, and in Overlay2 the myc-TR3, RXRα, and Hsp60 images are overlaid. **(E) Mitochondrial localization of RXRα is TR3 dependent.** LNCaP cells or LNCaP cells stably expressing TR3 antisense RNA (TR3/Antisense) were treated with or without TPA for 1 hr, then immunostained with anti-RXRα antibody. **(F) Effect of RXR siRNA on TR3 localization.** LNCaP cells were transfected with or without RXRα siRNA or control siRNA for 72 hr. Cells were analyzed for subcellular localization of RXRα and TR3 by confocal microscopy.

Identification of a putative NES in RXRα. To study whether the cytoplasmic localization of the RXRα/TR3 heterodimer is mediated through CRM1-dependent nuclear export we examined the effect of the inhibitor leptomycin B (LMB) on this type of export. Treatment of LNCaP cells with LMB completely blocked TPA-induced cytoplasmic localization of TR3 and RXRα (Figure 3A). This result suggests that the nuclear export of RXRα/TR3 heterodimer is mediated by the CRM1-dependent mechanism.

CRM1-dependent nuclear export is mediated by a Leucine-rich nuclear export signal (NES). Our mutagenesis studies revealed the presence of a Methionine-rich sequence, which has significant homology to various identified NESs (Figure 3B). The DNA sequences representing RXRα residues 348-368 were fused to green fluorescent protein (GFP), and the fusion was transfected into HEK293T embryonic kidney cells. HEK293T cells were chosen for our mechanistic studies because of their high transfection efficiency. The GFP-NES fusion was found exclusively in the cytoplasm, while LMB treatment prevented its nuclear export. Mutations of Met357 and Met360 to Ala (GFP-NESm-RXRα) largely abolished nuclear export activity (Figure 3C). Thus, RXRα amino acids 348-368 represent a putative NES.

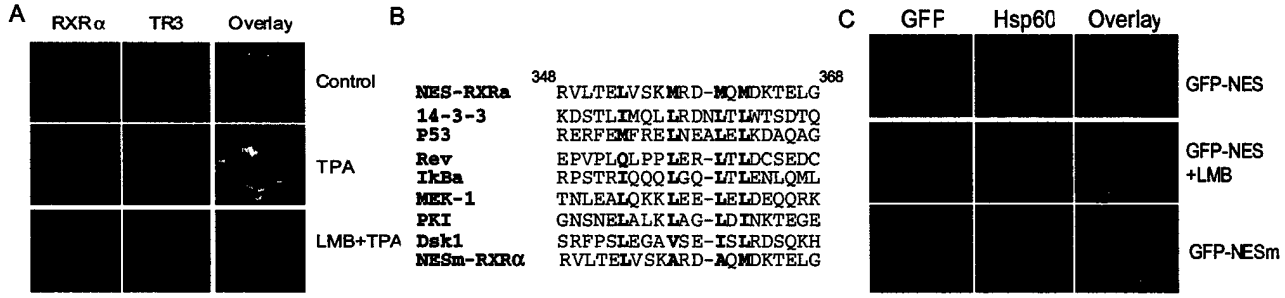

Figure 3. Identification of a nuclear export sequence in RXRα. (A) Cytoplasmic localization of RXRα/TR3 is mediated by CRM1-dependent nuclear export. LNCaP cells were treated with TPA in the absence (control) or presence of leptomycin B (LMB, 2.5 ng/ml) (Sigma) and analyzed by confocal microscopy. **(B)** Schematic representation of the RXRα NES. The identified RXRα NES is compared with known NESs identified in the indicated genes. The bold letters indicate conserved amino acid residues. **(C)** The RXRα NES is capable of directing GFP to the cytoplasm. The putative RXRα NES (RVLTELVSKMRDMQMDKTELG) or its mutant (RVLTELVSKARDAQMDKTELG) (NESm) was fused to GFP, and the expression vectors were transfected into HEK293T embryonic kidney cells. Cells were treated with or without LMB for 6 hr, then stained for Hsp60 and analyzed by confocal microscopy.

Regulation of the RXRα NES activity by ligand binding. To determine whether RXRα ligands inhibited RXRα NES activity through their induction of RXRα dimerization, we examined their effects on the cytoplasmic accumulation of RXRα/C1, a mutant RXRα ligand-binding domain that exclusively localized in the cytoplasm of HEK293T cells (data not shown). Treatment of GFP-RXRα/C1-transfected cells with RXRα ligand 9-*cis*-RA or SR11237 resulted in the diffuse distribution of RXRα/C1 throughout the cells, whereas treatment the RARα subtype-selective Am80 produced no such effect (Figure 4A). Nondenaturing

polyacrylamide gel electrophoresis (PAGE) indicated that GFP-RXRα/C1 occurred only as a monomer in the cytoplasmic fraction of these cells. After treatment with 9-*cis*-RA, the monomeric form of GFP-RXRα/C1 in the cytoplasmic fraction disappeared, and only the homodimeric form was detected in the nuclear fraction of the lysate (Figure 4B). Thus, monomeric RXRα exists in the cytoplasm, while liganded-homodimeric RXRα is in the nucleus. RXR ligands 9-*cis*-RA and SR11237 inhibit RXRα nuclear export probably by inducing RXRα homodimerization.

We also analyzed the subcellular localization of two homodimerization defective-RXRα mutants, RXRα/385, which lacks the major homodimerization domain, and RXRα/LLL, which has Leu418, Leu420, and Leu430 in helix 10 replaced with Ala and fails to homodimerize in response to 9-*cis*-RA (8). Interestingly, both RXRα mutants were exclusively localized in the cytoplasm regardless of the presence of 9-*cis*-RA (Figure 4C). Another RXRα mutant, RXRα/C1/C432A, which also fails to homodimerize in response to 9-*cis*-RA (data not shown), was stained exclusively in the cytoplasm despite 9-*cis*-RA treatment (Figure 4C). Thus, subcellular localization of RXRα depends on its dimerization status and homodimerization suppresses RXRα nuclear export.

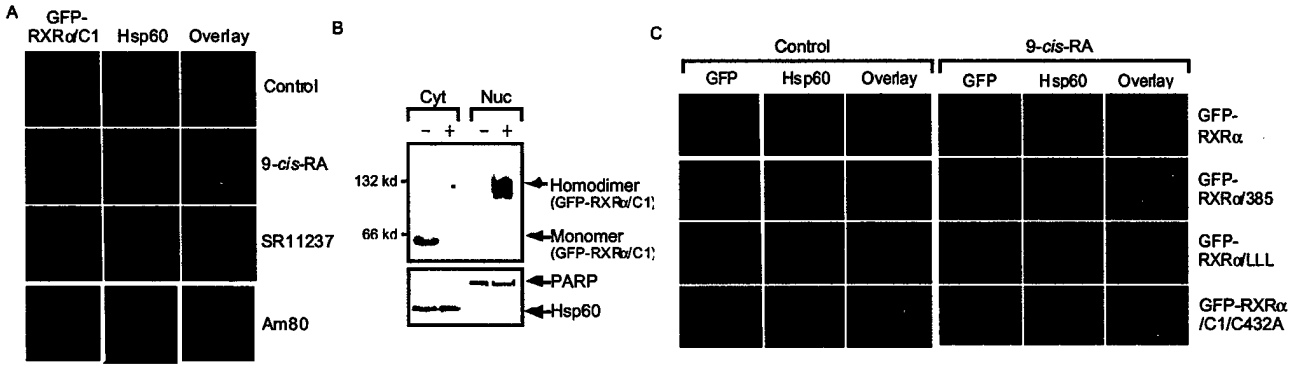

Figure 4. Regulation of RXRα nuclear export by its ligands and homodimerization. (A). Analysis of subcellular localization of RXRα/C1 in the absence or presence of retinoids. GFP-RXRα/C1 was transfected into HEK293T cells, which were then treated with the indicated retinoid, stained with Hsp60, and analyzed by confocal microscopy. **(B).** RXRα/C1 dimerization status determines its subcellular localization. GFP-RXRα/C1 was transfected into HEK293T cells, which were not treated or treated with 9-*cis*-RA (10^{-7}M). Nuclear and cytoplasmic extracts were prepared and analyzed by nondenaturing PAGE and anti-GFP antibody. The same extracts were analyzed by denaturing PAGE for expression of PARP and Hsp60 to ensure nuclear and mitochondrial fraction purity, respectively. **(C).** Confocal microscopy analysis of RXR homodimerization-defective mutants. GFP-RXRα/385, GFP-RXRα/LLL or GFP-RXRα/C1/C432A was transfected into HEK293T cells. Cells were treated with or without 9-*cis*-RA and analyzed by confocal microscopy.

Unique TR3/RXRα heterodimerization. Heterodimerization of RXRα with RARα in solution largely depends on their dimerization interfaces localized in their LBDs and has been mapped to a region in the carboxyl (C)-terminus, corresponding to helices 9 and 10 in the canonical nuclear receptor LBD structure (9, 10). In GST pull-down assays, full-length RXRα was pulled-down by either GST-TR3 or GST-RARα, whereas RXRα/385 was only pulled-down by GST-TR3 but not by GST-RARα (Figure 5B). Thus, in solution the RXRα C-terminus is required for its interaction with RARα but not with TR3. Similarly, as TR3/467, a mutant lacking the C-terminus, was effectively pulled-down by GST-RXRα (Figure 5C), the TR3 C-terminus was also dispensable for TR3 interaction with RXRα in solution. *In vivo* co-immunoprecipitation showed the efficient precipitation of GFP-RXRα/385 by anti-Flag antibody when Flag-TR3 was coexpressed in HEK293T cells (Figure 5D). In transactivation reporter gene assays, both RXRα/385 and RXRα similarly inhibited TR3 homodimer activity in the absence of 9-*cis*-RA (Figure 5E). Interestingly, TR3/467 and RXRα/385 colocalized in cells (Figure 5F). In addition to the LBD dimerization interface, RXRα contains another dimerization interface in the DNA-binding domain (DBD). Interestingly, TR3 also has a unique dimerization interface in its DBD called the C-terminal extension (11). Together, these results demonstrate that the C-termini of RXRα and TR3 are not required for their interaction in solution. Our results suggest that RXRα/TR3 heterodimerization in solution is mediated by the DBD dimerization interfaces (see also Figure 11). Such a unique heterodimeric structure may allow the activation of the RXRα NES.

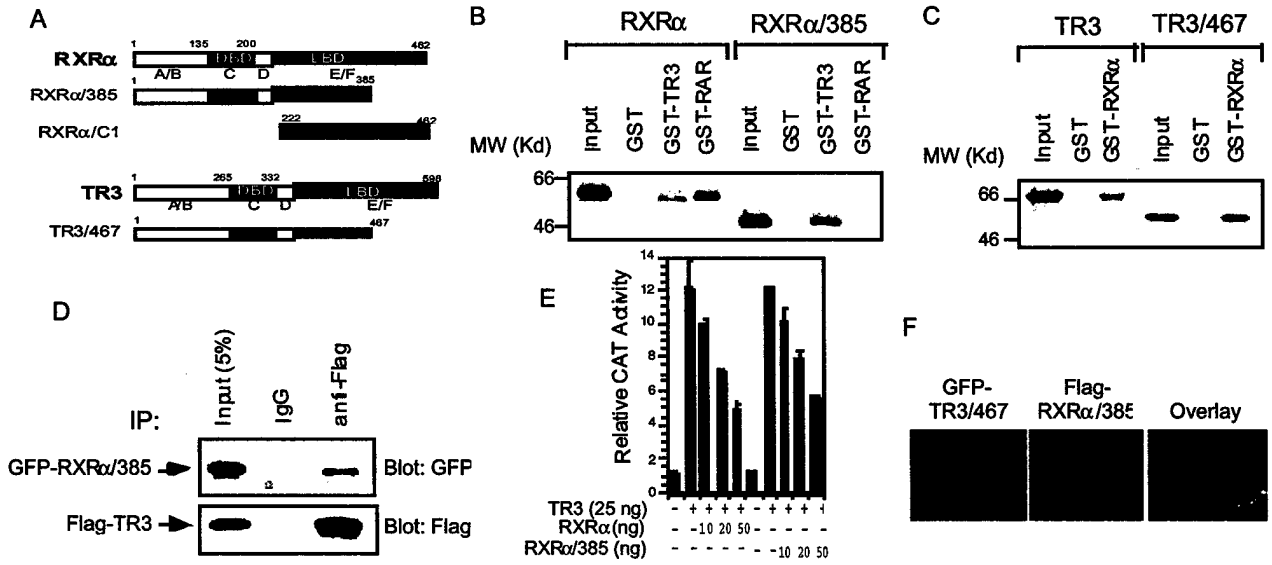

Figure 5. C-termini of RXRα and TR3 are not required for RXRα/TR3 interaction in solution. **(A)** Schematic representation of RXRα and TR3 mutants. The DBD, LBD, and A-F domains are indicated. **(B/C)** GST-pull-down assays for determination of RXRα/TR3 heterodimerization. GST-TR3, GST-RXRα or GST control protein immobilized on glutathione-Sepharose (20 l) was incubated with *in vitro* synthesized ^{35}S-labeled TR3 or RXRα (10 l) as indicated. Bound proteins were analyzed by SDS-PAGE/autoradiography. **(D)** Co-IP assay for TR3 and RXRα/385 interaction. Expression vectors for Flag epitope tagged-TR3 (Flag-TR3) and GFP-RXRα/385 were cotransfected into HEK293T cells. The expressed Flag-TR3 and GFP-RXRα/385 were then immunoprecipitated by using either anti-Flag antibody or control IgG, and immunoprecipitates were examined by western blotting using anti-GFP antibody. The same membranes were also blotted with anti-Flag antibody to determine precipitation specificity and efficiency. Input represents 5% of the total cell extract used in the precipitation assays. **(E)** Reporter gene assay. $(NurRE)_2$-*tk*-CAT (100 ng), β-galactosidase (100 ng) and TR3 (25 ng) expression vectors were transiently transfected into HEK293T cells together with or without RXRα or RXRα/385. Cells were treated with or without 9-*cis*-RA (10^{-7} M) as indicated. CAT activity was determined and

normalized relative to β-galactosidase (β-gal) activity. **(F)** RXRα/385 and TR3/467 colocalize in cells. GFP-TR3/467 and Flag-RXRα/385 were cotransfected into HEK293T cells, and their localizations were visualized by confocal microscopy.

Modulation of RXRα/TR3 heterodimerization by RXR ligands. The RXR/TR3 heterodimer binds and activates DNA sequences consisting of AGGTCA or like motifs arranged as a direct repeat with a 5-bp spacing (the DR-5 response element) (12). To characterize RXRα/TR3 heterodimerization on DNA, gel-shift assays were conducted using the βRARE, a DR-5 element, as a probe (Figure 6A). *In vitro* synthesized TR3 and RXRα bound to the βRARE as a heterodimer. However, when TR3/467, which heterodimerizes with RXRα in solution (Figure 5C), was incubated with RXRα, we did not detect any heterodimer binding to the βRARE (Figure 6A). Similarly, coincubation of RXRα/385 and TR3 did not result in formation of a stable heterodimer on the βRARE. Thus, the C-termini of both TR3 and RXRα are required for the formation of a stable RXRα/TR3 heterodimer complex on the βRARE. Consistently, TR3/467 failed to activate the βRARE on cotransfection with RXRα in either the absence or presence of RXR ligand SR11237, while cotransfection of full-length TR3 and RXRα strongly activated the βRARE in response to SR11237 (Figure 6B).

To determine how RXR ligands influence RXRα/TR3 heterodimer binding to the βRARE, RXRα protein was exposed to its ligand SR11237 or 9-*cis*-RA prior to incubation with TR3. Although the RXRα/TR3 heterodimer bound to the βRARE in the absence of an RXR ligand, the addition of SR11237 or 9-*cis*-RA strongly enhanced binding by the heterodimer (Figure 6C). These data suggest that RXRα/TR3 heterodimers in solution may be incompetent for DNA binding and that RXR ligand binding may induce a C-terminal-stabilized heterodimer conformation that favors DNA binding. In GST pull-down assays, incubation of GST-RXRα with 9-*cis*-RA strongly reduced the ability of GST-RXRα to interact with TR3/467 (Figure 6D), suggesting that 9-*cis*-RA binding may mask or alter the DBD dimerization interface required for RXRα binding to TR3 in solution. In comparison, the addition of 9-*cis*-RA did not affect the binding of RXRα/385 to GST-TR3. Thus, the dimerization interface for the formation of the RXRα/TR3 heterodimer in solution is different from that required for the formation of the DNA-bound heterodimer and is regulated by RXR ligand binding (Fig. 11).

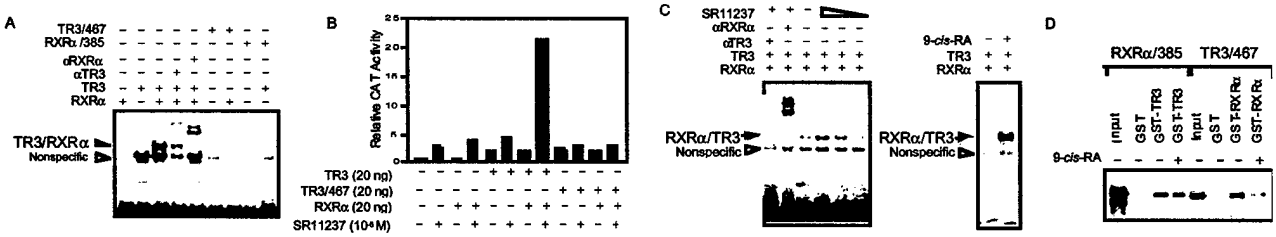

Figure 6. 9-*cis*-RA modulates RXRα/TR3 heterodimerization and promotes DNA binding. **(A)** The C-terminal domains of TR3 and RXRα are required for the formation of RXRα/TR3 heterodimer on DNA. The indicated TR3, RXRα or their mutants were synthesized *in vitro* and analyzed for binding to the βRARE by gel-shift assays. **(B)** The TR3 C-terminus is required for transactivation of the βRARE. βRARE-*tk*-CAT (100 ng), β-gal expression vector (100 ng) and the expression vector for TR3 or a TR3 mutant (20 ng) were transiently transfected into HEK293T cells with or without the RXRα expression vector. CAT activity was determined and normalized relative to β-gal activity. **(C)** Effect of RXR ligands on binding of RXRα-TR3 heterodimers to the βRARE. Equivalent amounts of *in vitro* synthesized TR3 and RXRα were incubated alone or together with or without RXR ligand SR11237 and analyzed by gel-retardation assays using the βRARE as a probe. **(D)** RXR ligand modulates RXRα/TR3 heterodimerization in solution. GST-pull-down assays for determination of RXRα/TR3 heterodimerization. The indicated GST fusions immobilized on

glutathione-Sepharose (20 l) were incubated with *in vitro* synthesized ^{35}S-labeled receptor protein (10 l). Bound proteins were analyzed by SDS-PAGE/autoradiography.

RXRα mitochondrial localization is inhibited by RXRα ligands. Inhibition of RXRα mitochondrial localization by its ligands was demonstrated by immunoblotting of mitochondria-enriched heavy membrane (HM) fractions from LNCaP cells. The accumulation of RXRα in the HM fraction after cells were treated with TPA or an analog of AHPN/CD437 (SR11453) was inhibited by pretreatment with 9-*cis*-RA (Fig. 7A). Similar to its effect on RXRα, mitochondrial accumulation of TR3 was also abolished by 9-*cis*-RA pretreatment (Figure 7A). Consistently, cytochrome *c* (cyt *c*) release (Figure 7B) and apoptosis (Figure 7C) were inhibited by RXR ligands in LNCaP cells.

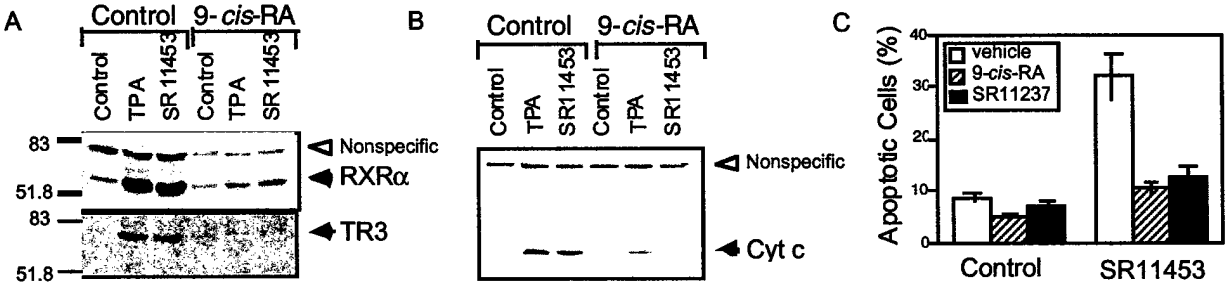

Figure 7. Effect of RXR ligands on RXR mitochondrial targeting and apoptosis. (A) Effect of 9-*cis*-RA on mitochondrial localization of RXRα and TR3. HM fractions were prepared from LNCaP cells treated with or without TPA or SR11453 (10^{-6} M) for 3 hr with or without a 9-*cis*-RA (10^{-7} M) pretreatment for 12 hr, and analyzed for expression of TR3 or RXRα by immunoblotting. **(B)** Inhibition of cyt *c* release by 9-*cis*-RA. LNCaP cells were treated with or without 9-*cis*-RA (10^{-7} M) for 12 hr before treatment with TPA (100 ng/ml) or SR11453 for 1 hr. Cytosolic fractions were analyzed for Cyt *c* by immunoblotting. A nonspecific band at ~ 70 kd served as a control for equal loading of proteins. **(C)** RXR ligands prevent SR11453 induced apoptosis. LNCaP cells were pretreated with 9-*cis*-RA or SR11237 for 12 hr before treatment with SR11453 for 48 hr. Apoptosis was determined by nuclear staining with DAPI.

2. Mitochondrial receptor of TR3

Interaction of TR3 with Bcl-2. We investigated the possibility that TR3 targeted mitochondria by binding to Bcl-2 that is known to reside on the mitochondrial outer membrane (13). GST pull-down assays showed that ^{35}S-labeled Bcl-2 was pulled-down by GST-TR3 but not by GST. Conversely, ^{35}S-labeled TR3 bound specifically to GST-Bcl-2 but not to the control GST protein (Figure 8A). We also conducted the mammalian two-hybrid assay to assess the interaction. The trans-membrane domain (TM) was deleted from Bcl-2 in order to prevent its membrane accumulation. The mutant (Bcl-2/ΔTM) fused with the Gal4-DNA-binding domain strongly activated a reporter containing the Gal4-binding site when it was cotransfected with Gal4 transactivation-domain fused with either TR3 or TR3/ΔDBD, a TR3 mutant lacking its DNA-binding domain (DBD) (Figure 8B). The interaction between TR3 and Bcl-2 was also demonstrated by the reporter gene assay, showing that transactivation of TR3 on its DNA responsive element was potently inhibited by cotransfection of Bcl-2 but not by Bax (Figure 8C). To determine whether TR3 and Bcl-2 colocalized in cells, we treated cells with TPA, which induces expression of endogenous TR3 and its mitochondrial localization in LNCaP cells. Confocal microscopy analysis demonstrated that, while a low level of TR3 was detected in the nucleus of LNCaP cells in the absence of TPA, the distribution patterns of TPA-induced TR3 and Bcl-2 overlapped extensively in the cytoplasm (Figure 8D). We also determined whether transfected TR3 and Bcl-2 colocalized in cells. TR3/ΔDBD, which constitutively resides on mitochondria in LNCaP cells, was fused to GFP. Confocal microscopy analysis demonstrated that

GFP-TR3/ΔDBD displayed a distribution pattern that overlapped with that of transfected Bcl-2, while the control GFP protein was diffusely distributed in cells (Figure 8E). To further confirm the interaction between TR3 and Bcl-2, we generated a monoclonal antibody against the ligand-binding domain (LBD) of TR3 for use in the co-immunoprecipitation (Co-IP) assay. LNCaP cells were treated with TPA to induce TR3 expression. Cell extracts were prepared from TPA-treated and nontreated cells and incubated with anti-TR3 antibody. As shown in Figure 8F, Bcl-2 was specifically coprecipitated by anti-TR3 antibody in TPA-treated cells, but not in nontreated cells. Together, these data demonstrate that TR3 interacts with Bcl-2.

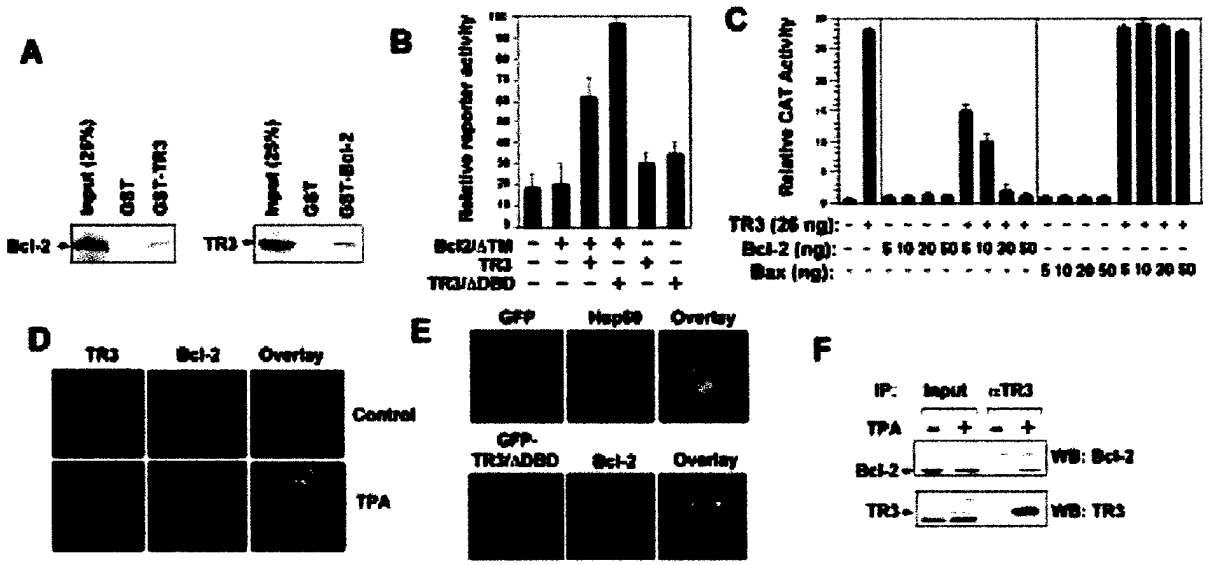

Figure 8. Interaction of TR3 with Bcl-2. **(A)** GST-pull down. GST-Bcl-2, GST-TR3 or GST control protein immobilized on 20 l of glutathione-Sepharose was incubated with 10 l of *in vitro* synthesized ^{35}S-labeled TR3 or Bcl-2 as indicated. Bound proteins were analyzed by SDS-PAGE autoradiography. **(B)** Mammalian two-hybrid assay. The trans-membrane (TM) domain was deleted from Bcl-2 (Bcl-2/DTM) to prevent its membrane accumulation for these experiments. Gal-4 reporter gene (Gal-4-*tk*-Luc, 250 ng) and 50 ng of β-galactosidase expression vector were co-transfected into CV-1 cells with Bcl-2/ΔTM fused with the Gal-4 DNA-binding domain (DBD) together with or without TR3 or TR3/ΔDBD fused with the Gal-4 transactivation domain. Reporter gene activity was determined 24 h after transfection and was normalized relative to β-galactosidase activity. **(C)** Inhibition of TR3-dependent transactivation by Bcl-2. CV-1 cells were transfected with the NurRE-*tk*-CAT (100 ng) and β-galactosidase expression vector (100 ng) with or without TR3 expression vector (25 ng) together with or without the indicated amount of Bcl-2 or Bax expression vector. CAT activity was then determined 24 h after transfection and normalized relative to β-galactosidase activity. The bars in **B** and **C** are means – S.D. from three and six experiments, respectively. **(D)** Confocal microscopy analysis for co-localization of endogenous TR3 and Bcl-2. LNCaP cells were treated with or without TPA for 3 h, then immunostained with anti-Bcl-2 antibody followed by FITC-conjugated secondary antibody, or with mouse monoclonal anti-TR3 antibody followed by Cy3-conjugated secondary antibody. Bcl-2 and TR3 were visualized using confocal microscopy and the images were overlaid (Overlay) as indicated. **(E)** Confocal microscopy analysis for colocalization of transfected GFP-TR3/ΔDBD and Bcl-2. Expression vectors for GFP—TR3/ΔDBDand Bcl-2 were cotransfected into LNCaP cells. After 20 h, cells were immunostained with anti-Bcl-2 antibody followed by Cy3-conjugated secondary antibodies (Sigma). GFP—fusion and Bcl-2 were visualized using confocal microscopy, and the two images were overlaid. As a control, the cellular distribution of transfected GFP empty vector is shown. **(F)** *In vivo* co-immunoprecipitation assay. LNCaP cells were treated with or without TPA for 3 hr to induce TR3 expression, and cell lysates were incubated with mouse

monoclonal anti-TR3 antibody. After immunoprecipitation, Western blotting was conducted by using anti-Bcl-2 antibody (Santa Cruz) or rabbit polyclonal anti-TR3 anti-body (Active Motif).

Bcl-2 mediates TR3 mitochondrial targeting and apoptosis. We studied whether mutant TR3/ΔDBD lacking the DNA-binding domain targets mitochondria by its interaction with Bcl-2. TR3/ΔDBD expressed in HEK293T cells had a diffuse distribution pattern (Figure 9A), but colocalized with Bcl-2 and heat shock protein 60 (Hsp60), a mitochondrial specific protein, when Bcl-2 and TR3/ΔDBD were coexpressed (Figure 9A). Immunoblotting of the mitochondria-enriched HM fractions showed a significantly enhanced accumulation of TR3/ΔDBD in mitochondria when Bcl-2 was coexpressed (Figure 9B). These results demonstrate that Bcl-2 acts as a receptor for TR3 to target mitochondria. We next studied the involvement of TR3/Bcl-2 interaction in TR3-induced cytochrome (cyt) *c* release. In the absence of Bcl-2 cotransfection, TR3/ΔDBD did not release cyt *c* from mitochondria as determined by confocal microscopy analysis (Figure 9C). However, after Bcl-2 cotransfection, TR3/ΔDBD and Bcl-2 colocalized and cyt c was released (Figure 9C). DAPI staining indicated that TR3ΔDBD only caused the apoptotic events of nuclear fragmentation and condensation when Bcl-2 was cotransfected (Figure 9D). Thus, TR3/ΔDBD-Bcl-2 interaction is required to induce cyt *c* release and apoptosis.

Our findings that Bcl-2 acts as a bridging factor allowing TR3 mitochondrial localization and apoptosis indicate that Bcl-2 does not act as an antiapoptotic protein in TR3-mediated apoptosis, but rather as a proapoptotic molecule. Our mutational analysis (data not shown) suggests that the TR3/Bcl-2 interaction converts Bcl-2 from an antiapoptotic to a proapoptotic molecule by inducing a Bcl-2 conformational change. This exciting finding further demonstrates that TR3 represents an ideal molecular target for developing cancer preventive and therapeutic drugs, since Bcl-2 is often overexpressed in cancer cells, including prostate cancer cells and is associated with multidrug resistance. In fact, overexpression of TR3 is associated with the favorable response of patients with diffuse large B-cell lymphoma to several chemotherapeutic agents (14).

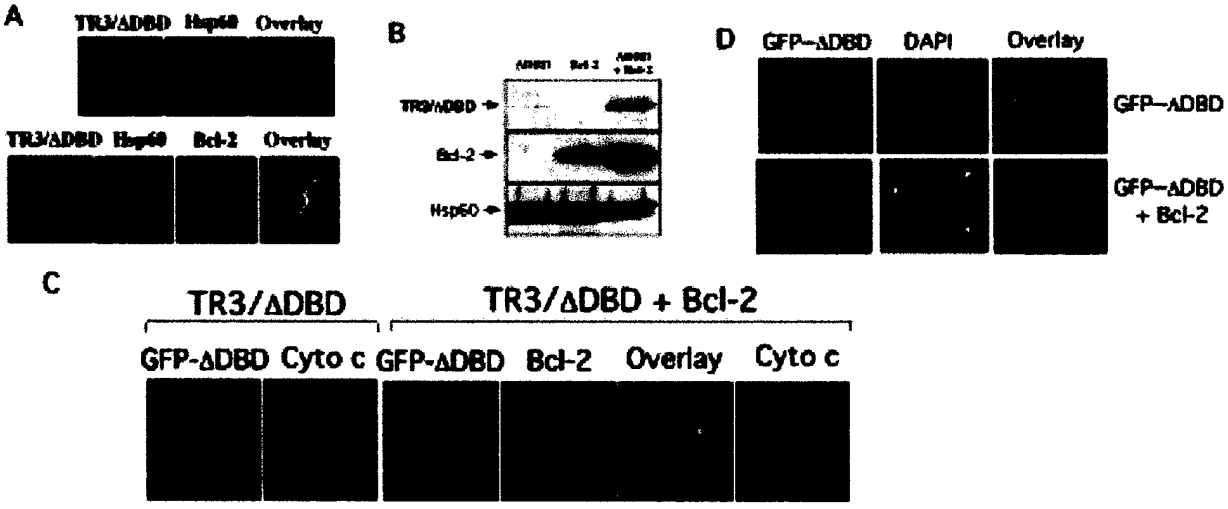

Figure 9. Bcl-2 expression promotes TR3/ΔDBD mitochondrial localization and its apoptotic effect. (A). Confocal microscopy analysis. GFP-TR3/ΔDBD and Bcl-2 were transfected into HEK293T cells alone or together. Cells were immunostained with anti-Bcl-2 antibody followed by Cy3-conjugated secondary antibody, or with anti-Hsp60 antibody followed by Cy5-conjugated

secondary antibody. Bcl-2, TR3/ΔDBD and mitochondria (Hsp60) were visualized using confocal microscopy. **(B)**. Western blotting. The heavy membrane (HM) fractions were prepared and analyzed for TR3/ΔDBD by Western blotting using anti-GFP antibody. **(C)**. Bcl-2 is required for TR3 to induce cyto *c* release. GFP-TR3/ΔDBD and Bcl-2 were transfected into 293T cells alone or together. Cells were immunostained with anti-cyto *c* (cyt *c*) antibody followed by Cy5-conjugated secondary antibody, or with anti-Hsp60 followed by Cy3-conjugated secondary antibody. Cyto *c*, TR3/ΔDBD, and mitochondria (Hsp60) were visualized using confocal microscopy. **(D)**. Bcl-2 is required for TR3/ΔDBD to induce apoptosis. Nuclei of cells transfected with GFP-TR3/ΔDBD alone or with Bcl-2 were stained by DAPI. GFP-TR3/ΔDBD expression and nuclear morphology were visualized by fluorescence microscopy. Arrows indicate cells displaying nuclear condensation and fragmentation.

3. Regulation of RARβ expression by RXR ligands.

We previously reported that RXR ligands could effectively induce RARβ expression in breast cancer cells (12). In studying how RXR ligands induce RARβ expression, we have reported that TR3/RXR heterodimer could bind effectively to the βRARE in the RARβ promoter and induced RARβ expression in response to RXR ligands. Interestingly, we also observed that RXR could bind to the βRARE as an RXR/PPARγ heterodimer (Figure 10A). Moreover, Western analysis showed that the RXR and PPARγ ligands were capable of inducing RARβ protein expression in ZR-75-1 and T-47D breast cancer cells (Figure 10B). Together, these data demonstrate that RXR ligands can induce RARβ expression through its activation of either RXR/TR3 or RXR/PPARγ heterodimer.

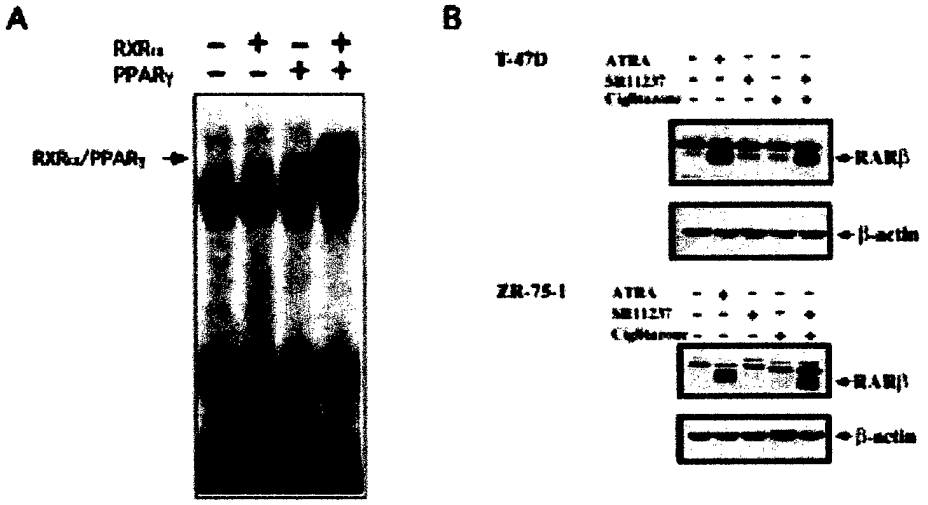

Figure 10. Regulation of RARβ expression by RXR and its ligands. A. RXR and PPARγ bind to βRARE as a heterodimeric complex. *In vitro* translated RXR and PPARγ were incubated with ^{32}P-radiolabelled βRARE either alone or in combination. The resulting reactions were then analyzed by EMSA. **B. Regulation of RARβ protein expression by RXR and PPARγ ligands in T-47D and ZR-75-1 breast cancer cells.** T-47D and ZR-75-1 cells were treated for 24 h with SR11237 (1 μM) in the absence or presence of ciglitazone (10 μM). Cell lysates were prepared and Western analysis performed as described in Materials and Methods.

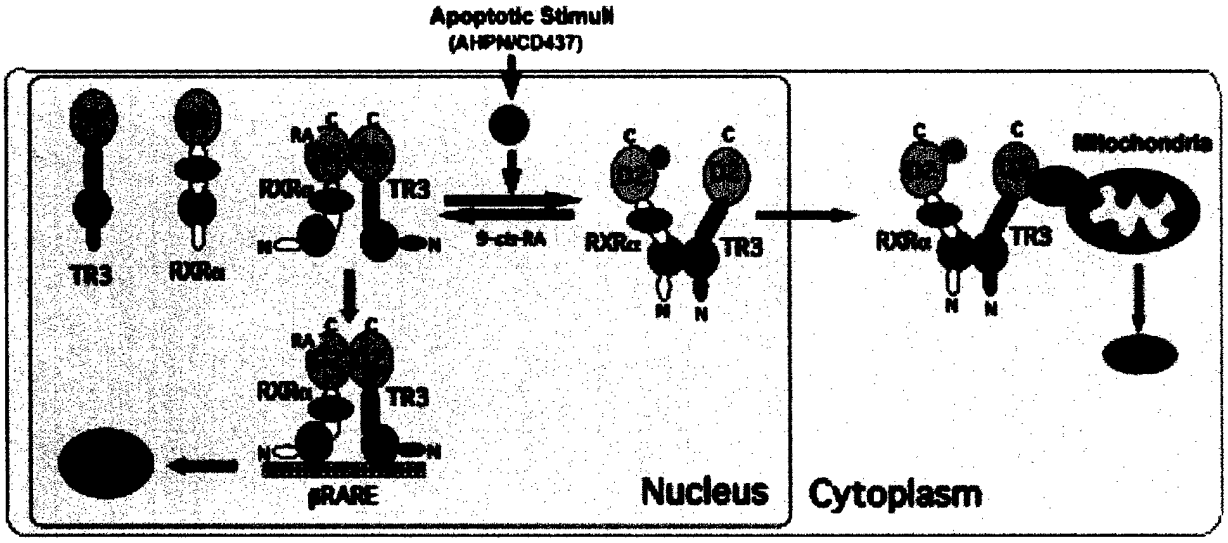

Figure 11. Mode of TR3 action supported by results obtained. Our results demonstrate that migratgion of TR3 from the nucleus to the cytoplasm requires its heterodimerization with RXRα. In response to an apoptotic stimulus, such as the retinoid AHPN, RXR or TR3 is modified probably through phosphorylation and then heterodimerize through their DNA-binding domain dimerization interfaces (D1), leading to the translocation of the RXR/TR3 heterodimer from the nucleus to the cytoplasm, where the heterodimer targets mitochondria through its interaction with Bcl-2. Mitochondrial targeting of the heterodimer then modulates mitochondrial function, resulting in the release of cytochrome *c* (cyt *c*) from the mitochondria into cytosol. In contrast, binding of RXR ligand 9-*cis*-RA to the RXR/TR3 heterodimer induces an RXR conformation that allows heterodimerization with TR3 through their ligand-binding domain interfaces (D2). Such a heterodimer favors DNA binding and may result in induction of RARβ, a potent growth inhibitor, through its binding to the βRARE in the RARβ promoter.

REPORTABLE OUTCOMES

1. Lin, F., Kolluri, S., Chen, G.-q.and Zhang, X.-k. Regulation of Retinoic Acid-induced Inhibition of AP-1 Activity by Orphan Receptor COUP-TF. ***J. Biol. Chem*** 277: 21414-22, 2002.

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CONCLUSIONS

We have investigated the mechanism by which retinoids induce apoptosis in breast cancer cells. Our results demonstrate that retinoid AHPN and its analogs exert their apoptotic effect by inducing migration of orphan receptor TR3 from the nucleus to mitochondria. In studying how the migration is regulated, we have observed that the migration of TR3 from the nucleus to the cytoplasm requires RXRα through their heterodimerization. We have also found that RXR contains a nuclear export sequence that is required for its cytoplasmic localization and is regulated by RXR ligands. Moreover, we have demonstrated a unique TR3/RXRα heterodimerization, which allows efficient nuclear export of the heterodimer. These data not only enhance our understanding the molecular mechanism by which TR3 translocates from the nucleus to the cytoplasm but also provide a molecular basis for developing RXR ligands that regulate apoptosis of breast cancer cells. In studying how TR3 targets mitochondria, we have discovered that TR3 can physically interacts with Bcl-2 and that Bcl-2 acts as a mitochondrial receptor of TR3 and is required for apoptotic effect of TR3 in breast cancer cells. Our finding that Bcl-2 is apoptotic when it interacts with TR3 provides novel approach to induce apoptosis of Bcl-2-expressing breast cancer cells. The overall plan for the next year of research remains the same as proposed in the original grant application.

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APPENDICES

Lin, F., Kolluri, S., Chen, G.-q.and Zhang, X.-k. Regulation of Retinoic Acid-induced Inhibition of AP-1 Activity by Orphan Receptor COUP-TF. ***J. Biol. Chem*** 277: 21414-22, 2002.

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Regulation of Retinoic Acid-induced Inhibition of AP-1 Activity by Orphan Receptor Chicken Ovalbumin Upstream Promoter-Transcription Factor*

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Retinoids are therapeutically effective in the treatment of various cancers, and some of the therapeutic action of retinoids can be ascribed to their potent inhibition of AP-1 activity that regulates transcription of genes associated with cell growth. We recently reported that the expression of orphan receptor chicken ovalbumin upstream promoter-transcription factor (COUP-TF) plays a role in mediating the growth inhibitory effect of *trans*-retinoic acid (*trans*-RA) in cancer cells. To gain insight into the molecular mechanism by which COUP-TF regulates *trans*-RA activity, we evaluated the effect of COUP-TF on antagonism of AP-1 activity by *trans*-RA. Our results demonstrated a positive correlation between COUP-TF expression and the ability of *trans*-RA to inhibit AP-1 activity in various cancer cell lines. In transient transfection assay, expression of COUP-TF strongly inhibited tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate-induced AP-1 transactivation activity and transactivation of c-Jun/c-Fos in both a *trans*-RA-dependent and -independent manner. *In vitro* studies demonstrated that the addition of COUP-TF inhibited c-Jun DNA binding through a direct protein-protein interaction that is mediated by the DNA binding domain of COUP-TF and the leucine zipper of c-Jun. Stable expression of COUP-TF in COUP-TF-negative MDA-MB231 breast cancer cells restored the ability of *trans*-RA to inhibit 12-*O*-tetradecanoylphorbol-13-acetate-induced c-Jun expression. The effect of COUP-TF in enhancing the *trans*-RA-induced antagonism of AP-1 activity required expression of retinoic acid receptors (RARs), since stable expression of COUP-TF in COUP-TF-negative HT-1376 bladder cancer cells, which do not express RARα and RARβ, failed to restore *trans*-RA-induced AP-1 repression. Thus, COUP-TF, through its physical interaction with AP-1, promotes anticancer effects of retinoids by potentiating their anti-AP-1 activity.

Retinoids, the natural and synthetic vitamin A analogs, exert profound effects on many biological processes, including cell proliferation and differentiation (1, 2), and are recognized as promising agents for the prevention and treatment of various cancers. The effects of retinoids are mainly mediated by two nuclear receptor classes, the retinoic acid receptor (RAR)[1] and retinoid X receptor (RXR) (3–5). Both are encoded by three different genes (α, β, and γ) and function as ligand-inducible transcription factors *in vivo* mainly as RXR/RAR heterodimers. *trans*-Retinoic acid (*trans*-RA) binds RARs, whereas 9-*cis*-RA binds both RARs and RXRs. Binding of retinoids to their receptors induces receptor conformational changes that switch on transcription of genes containing RA response elements (RAREs) (3–5). In addition to their positive regulation of RARE-containing genes, retinoid receptors, in response to their ligands, can inhibit effects induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the transcriptional activity of the proto-oncogenes c-Jun and c-Fos (6), which are components of the AP-1 complex, which often has a role in cancer cell proliferation (7). The activation of AP-1-responsive genes by TPA or c-Jun/c-Fos through TPA response elements (TREs) is repressed by retinoid receptors in a ligand-dependent manner (8–10). Conversely, AP-1 represses transactivation of retinoid receptors (8, 9). This mutual antagonism appears to play a critical role in regulating cell growth and differentiation (6). For example, overexpression of c-Jun conferred retinoid resistance to breast cancer cells (11), while overexpression of retinoid receptors enabled *trans*-RA to inhibit AP-1 activity in ovarian cancer cells and their growth (12). The functional interaction between AP-1 and retinoid receptors is also observed for other nuclear receptors, including glucocorticoid receptor (13–16), thyroid hormone receptor (17), vitamin D receptor, estrogen receptor (18, 19), and androgen receptor (AR) (18).

The anti-AP-1 activities shown by liganded retinoid receptors appear to contribute significantly to the therapeutic efficacy of retinoids against hyperproliferative diseases (6). Transcription of various AP-1-responsive genes, such as collagenase and stromelysin, which have roles in tumor progression and invasiveness (20), is inhibited by retinoids (8–10) and has been reported to contribute to their reversal of human bronchial epithelial squamous differentiation (21). Retinyl methyl ether, which effectively prevents mammary cancer in animals, strongly suppressed AP-1 activity in breast cancer cell (22). Interestingly, RARβ, a negative regulator of cancer cell growth (23), potently inhibits AP-1 activity and collagenase expression in both breast and lung cancer cells (24). Recent studies demonstrate that retinoids that specifically inhibit AP-1 activity but antagonize RAR transactivation on RAREs inhibited the growth of many different types of cancer cells (25–27). Anti-AP-1 retinoids inhibited squamous differentiation of human

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[1] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; RARE, RA response element; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; CBP, cAMP-response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione *S*-transferase.

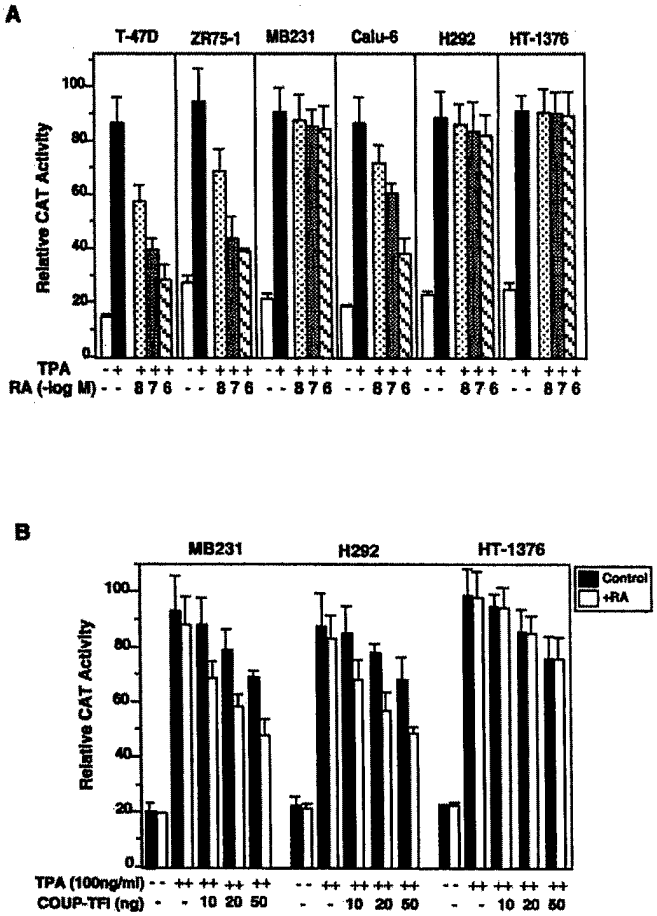

FIG. 1. **COUP-TF expression and *trans*-RA-induced anti-AP-1 activity correlate in cancer cell lines.** *A*, inhibition of TPA-induced collagenase promoter activity by *trans*-RA. The −73Col-CAT reporter was transfected into the indicated cancer cell lines. After transfection, the cells were incubated in DMEM medium containing 0.5% FCS for 24 h and treated with either TPA (100 ng/ml) alone or with the indicated concentrations of *trans*-RA. After 12 h, the cells were harvested, and CAT activity was determined. The activities of cotransfected β-galactosidase were used as controls for transfection efficiency. *B*, effect of COUP-TF expression in COUP-TF-negative cancer cell lines on inhibition of AP-1 activity by *trans*-RA. The −73Col-CAT reporter was transfected with or without COUP-TF into COUP-TF-negative cancer cell lines (MDA-MB231, H292, and HT-1376). Cells were incubated in medium (see "Experimental Procedures") containing 0.5% FCS for 24 h and then treated with either TPA (100 ng/ml) alone or with 10^{-6} M *trans*-RA. After 12 h, cells were harvested, and CAT activity was determined. The activities of cotransfected β-galactosidase were used as reference values.

bronchial epithelial cells (21), TPA-induced transformation and the clonal growth of the promotion-sensitive JB6 mouse epidermal cell line (28), and papilloma formation in animals (29). Thus, anti-AP-1 activity of retinoids contributes to their chemopreventive and chemotherapeutic effects, presumably by blocking the processes of tumor promotion and cell transformation.

The mechanism by which retinoids inhibit AP-1 activity remains largely unclear. Unlike the effect of retinoid receptors on RAREs, inhibition of AP-1 activity by retinoid receptors is independent of retinoid receptor-RARE interaction (8–10). Previous studies suggested several possible mechanisms for AP-1 inhibition by retinoid receptors. First, retinoid receptors are reported to physically interact with c-Jun and/or c-Fos (8, 9). This interaction results in the mutual inhibition of their DNA binding and transactivation functions and could explain the cross-talk occurring between AP-1 and retinoid signaling. However, a large excess of either retinoid receptor protein or c-Jun and c-Fos proteins was required to inhibit *in vitro* binding to the TRE or RARE, respectively (8, 9). Because the *in vivo* footprint assay for glucocorticoid receptor and AP-1 interaction did not reveal any effect on DNA binding (30), whether RAR and AP-1 directly interact *in vivo* remains to be established. Subsequently, it was suggested that blocking activation of the Jun N-terminal kinase signaling pathway that activates AP-1 might be responsible for AP-1 inhibition by retinoid and other nuclear receptors (31). Although this mechanism may explain how retinoids inhibit AP-1 activity under some conditions, it does not address how mutual inhibition occurs. Recent results suggest that the molecular basis of receptor-mediated inhibition of AP-1 transcriptional activation might be due to competition for a common coactivator, such as the cAMP-response element-binding protein (CBP), which is required for transcriptional activation by both the AP-1 complex and RARs (32). However, a domain of RAR capable of inhibiting AP-1 activity, such as the DNA-binding domain (33), does not interact with CBP (32). The development of retinoids that specifically inhibit AP-1 activity without transactivating RARs (25, 26) also argues against the involvement of RAR coactivators in RARs-AP-1 cross-talk. Thus, the availability of CBP is unlikely to be the sole modulator of RAR and AP-1-signaling pathways, and other adapter proteins may be involved in the antagonism of AP-1 activity by RARs.

Recent studies demonstrate that orphan receptor COUP-TF is involved in regulation retinoid responses (34, 35). COUP-TF is encoded by two distinct genes, COUP-TFI (EAR-3) (36, 37) and COUP-TFII (ARP-1) (38). Both show exceptional homology and overlapping expression patterns, suggesting their redundant functions (39). COUP-TF can modulate retinoid responses through either its high affinity binding to various RAREs or its heterodimerization with RXR (40–43). We previously reported that COUP-TF expression was required for cancer cell growth inhibition by *trans*-RA (35). We further demonstrated that the effect of COUP-TF is partly due to its induction of RARβ that mediates growth inhibition by retinoids in various cancer cells (35).

To further understand how COUP-TF is involved in the regulation of *trans*-RA activity in cancer cells, we investigated the effect of COUP-TF on antagonism of AP-1 activity by *trans*-RA. Our data demonstrate that COUP-TF expression in various cancer cell lines correlated with the ability of *trans*-RA to suppress AP-1 transcriptional activity. In addition, we found that COUP-TF effectively suppressed AP-1 transcriptional activity by interacting with c-Jun to cause loss of c-Jun DNA binding. This interaction required COUP-TF DNA-binding domain and the c-Jun leucine-zipper domain. Although AP-1 inhibition by COUP-TF did not require *trans*-RA, COUP-TF strongly potentiated the AP-1 antagonism by *trans*-RA when RARs were expressed. Our results demonstrate that interaction between COUP-TF and AP-1 is involved in regulating anti-AP-1 activity of retinoids in cancer cells and suggest that COUP-TF plays a role in the cross-talk between retinoid and AP-1 signalings.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa ovarian and MDA-MB231 breast cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS); Calu-6 lung cancer and HT-1376 bladder cancer cells were grown in MEM containing 10% FCS; and T-47D and ZR-75-1 breast cancer and H292 lung cancer cells were grown in RPMI-1460 medium with 10% FCS.

Plasmid Construction—CAT reporter constructs TRE-tk-CAT and −73Col-CAT have been described (8, 17, 22, 24), as have expression vectors for RARα, COUP-TF, c-Jun, and c-Fos (8, 17, 22, 24, 44). pcDNA3-COUP-TFII C-terminal deletion mutants pcDNA3-COUP-TFIIΔ7, pcDNA3-COUP-TFIIΔ30, and pcDNA3-COUP-TFIIΔ108 were generated as described (35). pcDNA3-COUP-TFII N-terminal deletion mutants were

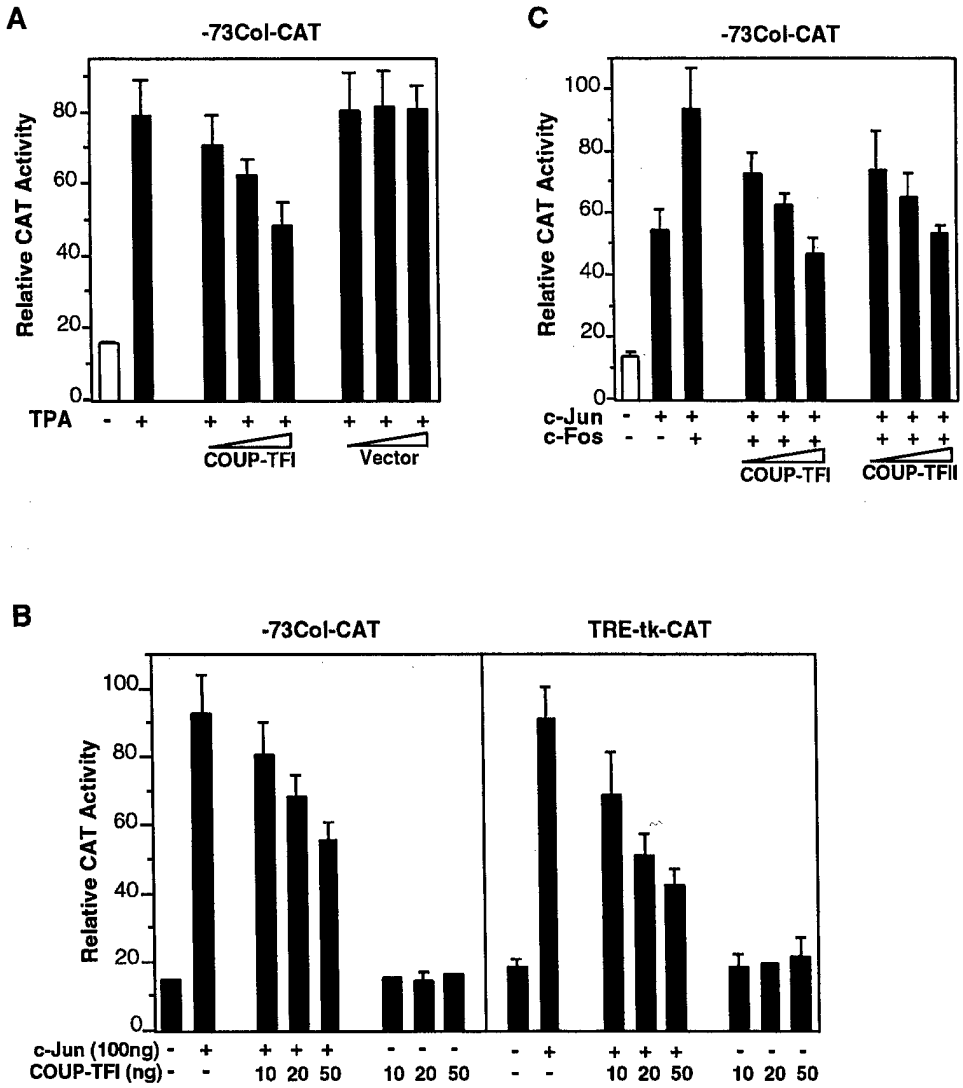

FIG. 2. **Inhibition of AP-1 activity by COUP-TF.** *A*, inhibition of TPA-induced collagenase promoter activity by COUP-TF in HeLa cells. The −73Col-CAT reporter was cotransfected without or with COUP-TFI expression vector (10, 20, or 50 ng) or the control pcDNA3 vector into cells. After transfection, cells were incubated in DMEM medium containing 0.5% FCS for 24 h and then treated with or without TPA (100 ng/ml). After 12 h, the cells were harvested, and CAT activity was determined. *B*, inhibition of c-Jun-induced −73Col-CAT and TRE-tk-CAT activity by COUP-TF in HeLa cells. The −73Col-CAT or TRE-tk-CAT reporter was cotransfected with/without c-Jun in the presence or absence of COUP-TFI expression vector (10, 20, and 50 ng) into cells. After transfection, cells were incubated in DMEM medium containing 0.5% FCS for 24 h, and CAT activity was determined. *C*, inhibition of c-Jun and c-Fos activities by COUP-TF in HeLa cells. The −73Col-CAT reporter was cotransfected with/without c-Jun (50 ng) and/or c-Fos (50 ng) expression vectors either in the presence or absence of COUP-TFI or COUP-TFII expression vector (10, 20, and 50 ng) into HeLa cells. The cells were then harvested, and CAT activity was determined.

constructed by cloning PCR products from COUP-TFII into pcDNA3 (Stratagene) using the following forward primers: TATAGGTACCATGGCGCCGCCCGTGCCC for pcDNA3-COUP-TFIIΔN25; ATATGGTACCATGACGCCAGCCCAGACG for pcDNA3-COUP-TFIIΔN50; AATTGGTACCATGCACATCGAGTGCGTG for pcDNA3-COUP-TFIIΔN75; and ATTAGGTACCATGCACCATCGCAACCAG for pcDNA3COUP-TFIIΔN125. The oligonucleotide GTGGCAGTTGAGGGGATCC was used as the reverse primer for these mutants.

Transient and Stable Transfection Assays—HeLa cells (1×10^5 cells/well) were plated in 24-well plates for 16–24 h before transfection as described (34, 35, 45), and other cancer cells (5×10^5 cell/well) were seeded in six-well plates. A modified calcium phosphate precipitation procedure was used for transient transfections (34, 35, 45). Briefly, 200 ng of reporter plasmid, 100 ng of β-galactosidase of expression vector (pCH 110; Amersham Biosciences), and various amounts of each expression vector were mixed with carrier DNA (pBluescript) to give 1000 ng of total DNA/well. CAT activity was normalized for transfection efficiency to the responding β-Gal activity. For stable transfections, the pRC/CMV-COUP-TF recombinant was transfected into MDA-MB231 and HT-1376 cells by the calcium phosphate precipitation method, and the stable clones were screened with G418 (Invitrogen) as described (35). Integration and expression of transfected cDNA were determined by Southern blotting and Northern blotting, respectively.

Preparation of Receptor Proteins—Receptor proteins for RARα, TR3, COUP-TF, and its mutants were synthesized by *in vitro* transcription-translation using rabbit reticulocyte lysates (Promega) as described previously (34). Amounts of translated proteins were determined by [^{35}S]methionine incorporation and SDS-PAGE with quantitation by incorporated radioactivity after normalization relative to methionine content.

Gel Retardation Assay—A fragment of the collagenase promoter region −73 to +63 containing one AP-1-binding site (TRE) was excised from a collagenase-CAT construct (8). In addition, the AP-1 binding sequence 5′-GATCCGGATGAGTCACCA-3′ was synthesized. Two fragments were labeled with [^{32}P]dCTP for use as probes for protein-DNA interaction. *In vitro* translated protein was incubated with the probe in a 20-μl reaction mixture containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC) at 25 °C for 15 min. DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels, and then gels were dried and analyzed by autoradiography.

GST Pull-down Assay—To prepare glutathione *S*-transferase (GST)–c-Jun or GST-c-Jun mutant fusion proteins, each c-Jun DNA or c-Jun mutant fragment was cloned in frame into the expression vector

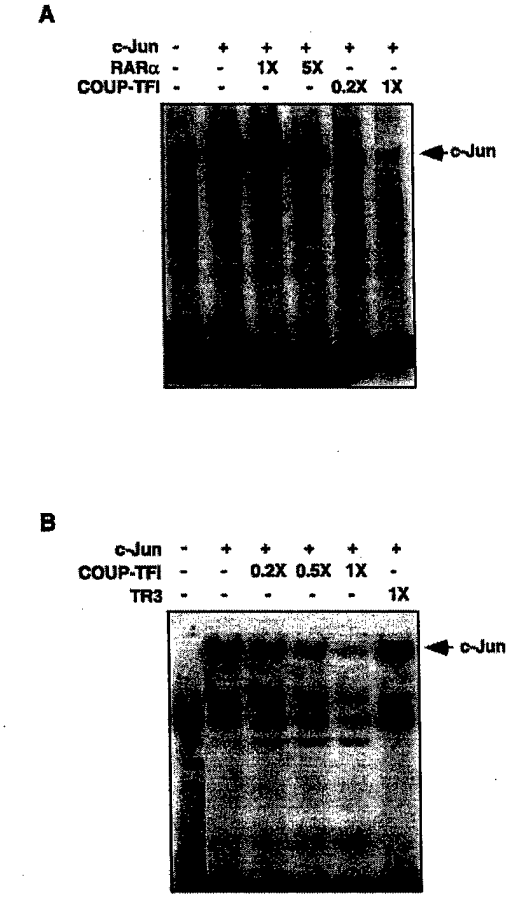

FIG. 3. **Inhibition of c-Jun binding to DNA by COUP-TF.** *In vitro* synthesized c-Jun was preincubated with the indicated molar excess of COUP-TF, RARα, or *TR3*. Unprogrammed reticulocyte lysate was added to maintain an equal protein concentration in each reaction. Following preincubation, the reaction mixtures were incubated with ^{32}P-labeled collagenase promoter (*A*) or the TRE (*B*) and analyzed by gel retardation. The *arrowhead* indicates the c-Jun-binding complex.

pGEX-3X (Amersham Biosciences). Fusion proteins were expressed in bacteria using the manufacturer's procedure and analyzed by gel retardation assays and Western blotting (data not shown). To determine the interaction between COUP-TF and c-Jun, the fusion proteins were immobilized on glutathione-Sepharose beads. The vector protein (GST), prepared under the same conditions as a control, was also immobilized. Beads were preincubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min. ^{35}S-labeled *in vitro* translated COUP-TF proteins (2–5 μl, depending on translation efficiency) were then added to the beads. The beads in 200 μl in EBC buffer (140 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM sodium orthovanadate, and 50 mM Tris, pH 8.0) were rocked continuously for 1 h at 4 °C. After washing 5 times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40), bound proteins were analyzed by SDS-PAGE and autoradiography.

Northern Blotting—For Northern analysis, total RNAs were prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (30 μg) from different cell lines treated with or without *trans*-RA (10^{-6} M) in the presence or absence of TPA (100 ng/ml) was analyzed by Northern blotting as described (46).

RESULTS

Correlation between COUP-TF Expression and Inhibition of AP-1 Activity by trans-RA—We recently reported that expression of COUP-TF is required for growth inhibition and apoptosis induction by *trans*-RA in various cancer cells (35). Since inhibition of AP-1 activity by retinoids is known to contribute to their anticancer effects, we studied whether COUP-TF expression was involved in the process. The effect of *trans*-RA on inhibiting AP-1 activity (Fig. 1*A*) was evaluated in COUP-TF–positive T-47D and ZR-75–1 breast cancer and Calu-6 lung cancer cell lines and in COUP-TF–negative MDA-MB231 breast cancer, H292 lung cancer, and HT-1376 bladder cancer cell lines (35). AP-1 activity induced by TPA was determined by transient transfection using the reporter −73Col-CAT, which contains a TRE that binds AP-1 (8). TPA-induced reporter activity was strongly inhibited in a *trans*-RA-dependent manner in the COUP-TF–positive ZR-75-1, T-47D, and Calu–6 cell lines. In contrast, *trans*-RA showed very little effect on TPA–induced −73Col-CAT activity in the COUP-TF–negative MDA-MB231, H292, and HT-1376 cell lines. Thus, COUP-TF expression correlates positively with the ability of *trans*-RA to inhibit AP-1 transcriptional activity.

Trans-RA-dependent and -independent Antagonism of AP-1 Activity by COUP-TF—The positive association between COUP-TF expression and *trans*-RA-induced anti-AP-1 activity suggested that COUP-TF was required for *trans*-RA to inhibit AP-1 activity and that the absence of COUP-TF expression in MDA-MB231, H292, and HT-1376 cells might be responsible for the lack of *trans*-RA activity. Therefore, we transiently transfected the COUP-TF expression vector and the −73Col-CAT reporter into the COUP-TF-negative cell lines, in which *trans*-RA did not inhibit AP-1 transcriptional activity (Fig. 1*B*). Upon COUP-TF transfection, *trans*-RA inhibited TPA-induced reporter activity in both MDA-MB231 and H292 cells in a COUP-TF concentration-dependent manner. Interestingly, COUP-TF transfection alone produced some inhibition of AP-1 activity. This observation suggested that COUP-TF inhibited AP-1 activity in both *trans*-RA–dependent and -independent manners in MDA-MB231 and H292 cells. When evaluated in HT-1376 cells, COUP-TF expression was able to inhibit AP-1 activity independently of *trans*-RA (Fig. 1*B*). However, it failed to confer the ability of *trans*-RA to inhibit TPA-induced reporter activity, even at high transfection levels, suggesting that *trans*-RA-dependent inhibition of AP-1 activity by COUP-TF is impaired in this cell line.

The above results suggested that COUP-TF alone inhibited AP-1 activity. We then examined the anti-AP-1 activity of COUP-TF in HeLa cells, in which TPA strongly induced the transcription of the transfected −73Col-CAT reporter. Similar to that observed in other cancer cell lines (Fig. 1*B*), COUP-TFI cotransfection inhibited TPA-induced reporter activity in a COUP-TFI concentration-dependent manner (Fig. 2*A*). Cotransfection of COUP-TFII produced almost identical results (data not shown). Activation of collagenase promoter by TPA occurs mainly through induction of AP-1 activity that activates the TRE in the promoter (7). We therefore examined whether COUP-TF expression also interfered with transactivation activity of c-Jun homodimer and c-Jun/c-Fos heterodimer. Cotransfection of −73Col-CAT with the c-Jun expression vector into HeLa cells led to about 5-fold induction of reporter expression (Fig. 2*B*), presumably due to activation of the collagenase promoter by the c-Jun homodimer. The c-Jun–induced −73Col-CAT reporter activity was repressed when COUP-TFI expression vector was cotransfected. Similarly, −73Col-CAT reporter activity induced by c-Jun/c-Fos heterodimers was inhibited by COUP-TFI and COUP-TFII (Fig. 2*C*). To determine that inhibition of collagenase promoter activity by COUP-TF was mediated by the TRE, we evaluated how COUP-TFI affected the TRE-tk-CAT reporter, which has a TRE sequence fused to the thymidine kinase promoter (Fig. 2*B*). Our result showed that cotransfection of COUP-TFI significantly inhibited c-Jun-induced TRE-tk-CAT activity (Fig. 2*B*). These results clearly demonstrate that COUP-TF inhibits transcriptional activity of c-Jun homodimer and c-Jun/c-Fos heterodimer and that the inhibition of AP-1 activity by COUP-TF on the TRE is respon-

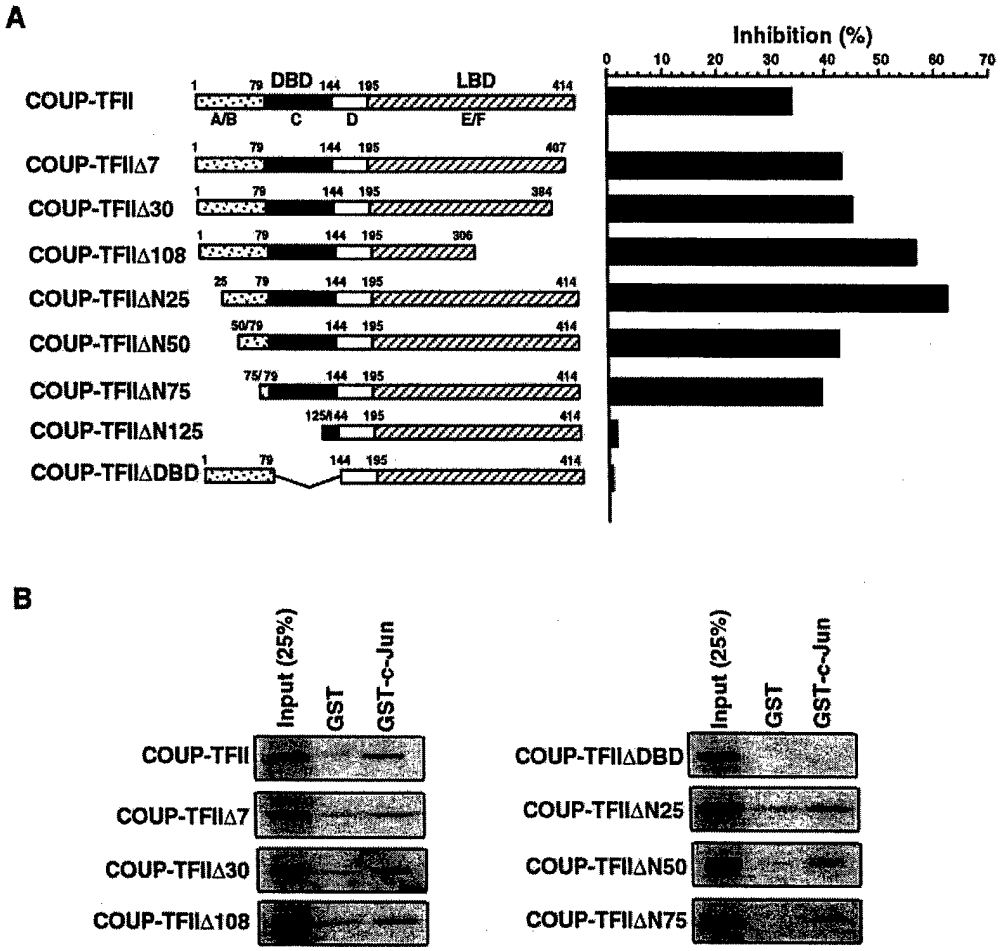

FIG. 4. **Interaction of c-Jun and COUP-TF and their domain requirements.** *A*, inhibition of transcriptional activity of AP-1 by COUP-TF mutants in HeLa cells. *Left*, schematic representation of COUP-TFII and its mutants. The DNA binding domain (*DBD*) and ligand-binding domain (*LBD*) of COUP-TFII are indicated. *Right*, effect of COUP-TFII mutants on AP-1 activity. The −73Col-CAT reporter was cotransfected without or with c-Jun (100 ng) alone or together with the indicated COUP-TFII mutants (50 ng). After transfection, cells were incubated in DMEM containing 0.5% FCS for 24 h. After 12 h, the cells were harvested, and CAT activity was determined. Reporter activity is shown as percentage of inhibition. *B*, interaction between c-Jun and COUP-TFs. c-Jun was synthesized in bacteria using pGEX-3X expression vector. GST-c-Jun fusion protein was immobilized on the glutathione-Sepharose beads. As a control, the same amount of glutathione *S*-transferase was also immobilized on the beads. *In vitro* translated ^{35}S-labeled COUP-TF and its mutant proteins were then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison.

sible for its antagonism of TPA activity.

Inhibition of c-Jun Binding to DNA by COUP-TF—Inhibition of AP-1 activity by several nuclear receptors has been shown to be due to their inhibition of AP-1 binding to DNA (8, 9, 13–15, 17, 22, 47). To study whether inhibition of AP-1 DNA binding by COUP-TF was involved in its inhibition of AP-1 activity, the purified collagenase promoter fragment was used as a probe in gel shift assays for binding of *in vitro* synthesized c-Jun protein in the absence or presence of COUP-TF protein (Fig. 3*A*). c-Jun alone formed a strong complex with the promoter. However, upon preincubation with COUP-TF protein, c-Jun binding was significantly inhibited. The addition of COUP-TF protein did not show any new complex formed with the collagenase promoter, indicating that the inhibitory effect of COUP-TF is not due to its direct binding to the collagenase promoter. We also studied the effect of COUP-TF on binding of c-Jun to the TRE. Preincubation with COUP-TF protein similarly inhibited c-Jun binding to the TRE derived from the collagenase promoter (Fig. 3*B*). As a comparison, preincubation of c-Jun with TR3 orphan receptor (34) that could not antagonize c-Jun transactivation (data not shown) did not inhibit c-Jun binding to the TRE. This result demonstrated that the inhibitory effect of COUP-TF on c-Jun binding to the collagenase promoter is not due to regions other than the TRE in the promoter. Next, we compared the inhibitory effect of COUP-TF with that of RARα that is known to repress AP-1 activity and inhibit c-Jun binding to TRE (8). Whereas an equal amount of COUP-TF significantly inhibited c-Jun binding to the collagenase promoter, an equal amount of RARα did not show any detectable inhibition (Fig. 3*A*). Inhibition of c-Jun binding required a 5-fold excess of RARα, similar to that observed before (8). The addition of *trans*-RA did not enhance the inhibitory effect of RARα (Ref. 8 and data not shown). Together, these results demonstrate that the inhibition of c-Jun binding to the TRE contributes to its suppression of AP-1 transcriptional activity by COUP-TF and that COUP-TF is a more effective inhibitor than RARα.

Interaction of c-Jun and COUP-TF—Our observation that COUP-TF inhibits transactivation and DNA binding by c-Jun prompted us to examine whether COUP-TF and c-Jun interact directly using GST pull-down assay (Fig. 4). *In vitro* synthesized radiolabeled COUP-TFII was specifically pulled down by bacterially expressed GST-c-Jun hybrid protein but not by GST protein (Fig. 4*B*), demonstrating that COUP-TF and c-Jun interact in solution. To identify the domain of COUP-TFII responsible for interacting with c-Jun, COUP-TFII deletion mutants (Fig. 4*A*) were constructed and analyzed on the −73Col-CAT reporter for antagonism of AP-1 activity in HeLa cells (Fig. 4*A*) and for their interaction with c-Jun using the

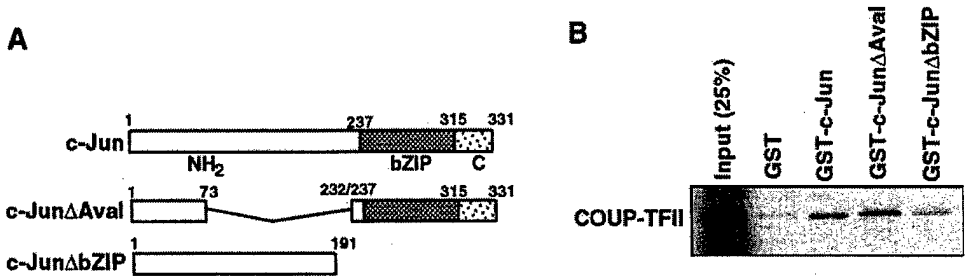

FIG. 5. **The domain of c-Jun required for interaction with COUP-TF.** *A*, schematic representation of c-Jun and its mutants. The amino terminus (NH_2), the basic region, the leucine zipper (*bZIP*), and the carboxyl terminus (*C*) are indicated. *B*, interaction between COUP-TF and c-Jun mutants. c-Jun and its mutant proteins were synthesized in bacteria using the pGEX-3X expression vector (Amersham Biosciences). GST-c-Jun and the mutant fusion proteins (GST-c-JunΔ*Ava*I and GST-c-JunΔbZIP) were immobilized on the glutathione-Sepharose beads. As a control, the same amount of glutathione transferase was immobilized on the beads. *In vitro* translated ^{35}S-labeled COUP-TFII was then mixed with the beads. After extensive washing, bound proteins were analyzed by SDS-PAGE. Input proteins are shown for comparison.

GST pull-down assay (Fig. 4*B*). Cotransfection of the c-Jun expression vector alone induced reporter transcription (Fig. 4*A*), whereas cotransfection with one of the C-terminal deletion mutants COUP-TFIIΔ7, COUP-TFIIΔ30, or COUP-TFIIΔ108 strongly inhibited c-Jun-induced reporter activity, as was observed using the wild-type COUP-TFII. N-terminal domain deletion mutants, such as COUP-TFIIΔN25, COUP-TFIIΔN50, and COUP-TFIIΔN75, also retained the inhibitory effect on AP-1 activity by COUP-TFII. In contrast, partial (COUP-TFIIΔN125) or complete deletion of the DNA-binding domain (COUP-TFIIΔDBD) abrogated anti-c-Jun activity completely. In the GST pull-down assay, COUP-TFII mutants that effectively suppressed AP-1 activity, such as COUP-TFIIΔ7, COUP-TFIIΔ30, COUP-TFIIΔ108, COUP-TFIIΔN25, COUP-TFIIΔN50, and COUP-TFIIΔN75, were pulled down by GST-c-Jun protein, whereas COUP-TFIIΔDBD, which failed to suppress AP-1 activity, was not (Fig. 4*B*). Thus, inhibition of AP-1 transcriptional activity by the COUP-TFII mutants correlated with their ability to interact with c-Jun. These observations further suggest that a direct c-Jun/COUP-TF interaction accounts for the inhibition of AP-1 activity by COUP-TF and that the DBD of COUP-TF is essential.

We also identified the region of c-Jun required for COUP-TF interaction (Fig. 5). Deletion of amino acids 73–232 from c-Jun (c-JunΔ*Ava*I) did not affect its ability to pull down COUP-TFII. In contrast, deletion of the C-terminal domain of c-Jun from amino acid 191 to 331 (c-JunΔbZIP), which encompasses the leucine-zipper region and basic region, completely abolished its ability to pull down COUP-TFII. Thus, the C-terminal domain, but not the N-terminal domain, of c-Jun is responsible for COUP-TF interaction.

Stable Expression of COUP-TF in COUP-TF-negative MDA-MB231 Cells Restores the Ability of trans-RA to Inhibit AP-1 Activity—To further examine the role of COUP-TF in *trans*-RA-induced inhibition of AP-1 activity, we analyzed the effect of *trans*-RA on TPA–induced c-Jun expression in MDA-MB231 breast cancer cells and MDA-MB231 cells stably transfected with COUP-TF (MB231/COUP#16) (35). Treatment of both cell lines with TPA strongly induced c-Jun expression as determined by Northern blotting (Fig. 6). Induction of c-Jun expression by TPA was probably due to an AP-1-binding site in the c-Jun promoter (7). Treatment with *trans*-RA did not affect basal c-Jun expression in both lines and did not inhibit TPA-induced c-Jun expression in MDA-MB231 cells. However, pretreatment of MB231/COUP#16 cells with *trans*-RA completely abolished TPA-induced c-Jun expression. These results demonstrate that COUP-TF expression is required for inhibition of AP-1 activity in MDA-MB231 cells by *trans*-RA.

Stable Expression of COUP-TF Does Not Lead to trans-RA-dependent Inhibition of AP-1 Activity in HT-1376 Cells Lacking

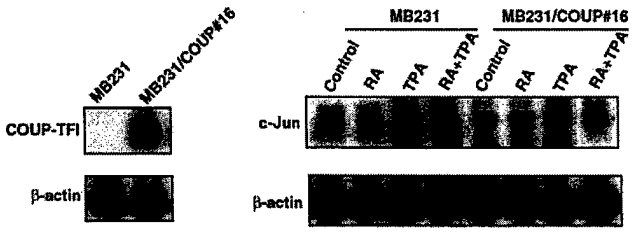

FIG. 6. **Stable expression of COUP-TF in COUP-TF-negative, RAR-positive MDA-MB231 cells restores the ability of *trans*-RA to inhibit AP-1 activity.** Total RNAs prepared from MDA-MB-231 or MDA-MB231 cells stably expressing COUP-TF (MB231/COUP#16) (35) treated with or without the indicated agents were analyzed for the expression of c-Jun. The expression of β-actin is shown to confirm the similar loading of RNA in each lane.

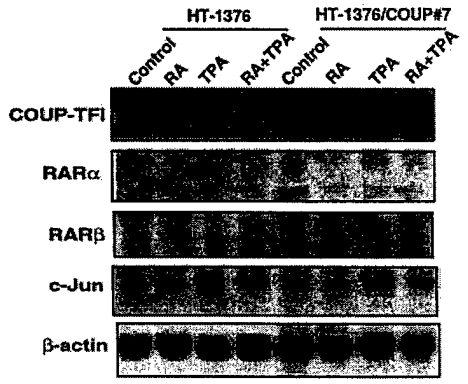

FIG. 7. **Stable expression of COUP-TF in COUP-TF and RAR-negative HT-1376 cells does not lead to *trans*-RA-dependent inhibition of AP-1 activity.** Total RNAs prepared from HT-1376 or HT-1376 cells stably expressing COUP-TF (HT-1376/COUP 7) treated with or without the indicated agents were analyzed for the expression of c-Jun, RARα, and RARβ. The expression of β-actin is shown to confirm similar loading of RNA in each lane.

RARα/β Expression—We also evaluated whether stable expression of COUP-TF affected TPA–induced c-Jun expression in HT-1376 bladder cancer cells (Fig. 7). The stable clone, HT-1376/COUP#7, which expresses high levels of COUP-TF, was analyzed for the effect of *trans*-RA on TPA activity (Fig. 7). About a 4-fold induction of c-Jun expression by TPA was observed in HT-1376 cells. However, the induction of c-Jun expression was largely reduced in HT-1376/COUP#7 cells, with only about a 2-fold induction. Interestingly, the basal level of c-Jun expression was also slightly reduced in HT-1376/COUP#7 cells. Despite the reduction of c-Jun expression in the absence or presence of TPA, *trans*-RA failed to inhibit basal and TPA-induced c-Jun expression in both wild-type and

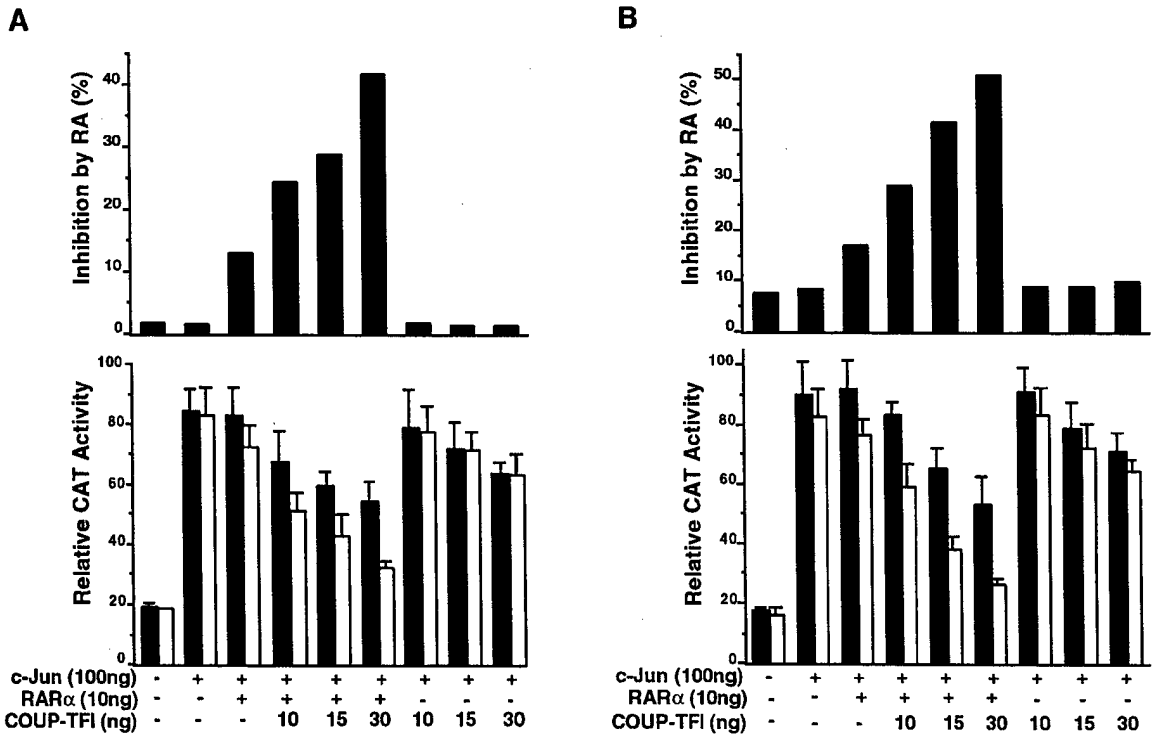

FIG. 8. **Effect of COUP-TF and RARα coexpression on antagonism of AP-1 activity by *trans*-RA.** The −73Col-CAT reporter was transfected together with c-Jun alone or together with COUP-TF and/or RARα expression vectors into HT-1376 (*A*) or HeLa (*B*) cells. After transfection, the cells were incubated in medium containing 0.5% FCS for 24 h and then treated with or without *trans*-RA. After 12 h, the cells were harvested, and CAT activity was determined. The *top panels* show the percentage of inhibition of c-Jun activity by *trans*-RA based on data shown in the *bottom panels*, in which *filled bars* represent control and *open bars* represent *trans*-RA treatment.

COUP-TF-expressing cells. Thus, COUP-TF expression is not sufficient to confer *trans*-RA-induced anti-AP-1 activity in HT-1376 cells.

RAR Expression Is Required for COUP-TF to Facilitate Antagonism of AP-1 Activity by trans-RA—Our observations that transient (Fig. 1*B*) or stable (Fig. 7) expression of COUP-TF in HT-1376 cells failed to modulate antagonism of AP-1 activity by *trans*-RA led us to investigate RAR expression. Unlike MDA-MB231 cells that express RARα (48) and RARβ when COUP-TF is expressed (35), HT-1376 cells did not express detectable RARα or RARβ (Fig. 7), although RARγ was expressed (data not shown). This result suggested that modulation of *trans*-RA-induced anti-AP-1 activity by COUP-TF might require RARα or RARβ. The effect of RARα expression on the ability of COUP-TF to regulate *trans*-RA activity was then examined using transient transfection in HT-1376 cells. The −73Col-CAT reporter was transfected into HT-1376 cells with or without c-Jun and COUP-TF and/or RARα vector. Transfected cells were treated with or without *trans*-RA. As shown in Fig. 8*A*, transfected COUP-TF repressed AP-1 activity in the absence of *trans*-RA. In contrast, transfected RARα (10 ng) led to inhibition of AP-1 activity in a *trans*-RA dependent manner. The addition of COUP-TF significantly enhanced the *trans*-RA-induced inhibition of AP-1 activity by RARα (Fig. 8*A*). Similar results were observed using HeLa cells (Fig. 8*B*). Thus, efficient inhibition of AP-1 activity by *trans*-RA activity by COUP-TF requires both COUP-TF and RARα.

DISCUSSION

Retinoids are effective growth inhibitors of cancer cells. Inhibition of AP-1 activity has been proposed as one mechanism by which retinoids exert their anticancer effects (6). Despite extensive studies in the last few years, how retinoids specifically antagonize AP-1 activity remains largely unknown. Here, we provide evidence that COUP-TF is involved in regulating the antagonism of AP-1 transactivational activity by *trans*-RA. COUP-TF, by physically interacting with c-Jun, inhibits AP-1 DNA binding and transactivation and is required for efficient inhibition of AP-1 activity by liganded RARs. Our results suggest that COUP-TF plays a role in the cross-talk between retinoid and AP-1 signaling pathways.

We recently reported that the expression of COUP-TF positively correlates with the inhibition of the growth of various cancer cell lines by *trans*-RA and that COUP-TF is underexpressed in many *trans*-RA-resistant cancer cell lines (35). Stable expression of COUP-TF in COUP-TF-negative cancer cells restores their sensitivity to *trans*-RA, demonstrating that COUP-TF can mediate anticancer effects of *trans*-RA (35). By studying anti-AP-1 activity of *trans*-RA in various cancer cell lines, we found a close correlation between COUP-TF expression and the anti-AP-1 activity of *trans*-RA (Fig. 1). In COUP-TF-positive ZR-75–1, T-47D, and Calu-6 cancer cell lines (35), *trans*-RA strongly inhibited the ability of TPA to activate transcription of the collagenase promoter, whereas in COUP-TF-negative MDA-MB231, H292, and HT-1376 cell lines, *trans*-RA failed to suppress TPA activity (Fig. 1). This finding is consistent with a previous study showing that *trans*-RA effectively inhibited AP-1 activity in ZR-75–1 and T-47D cells but not in MDA-MB231 cells (49). The requirement of COUP-TF in *trans*-RA-mediated AP-1 inhibition was further demonstrated by our findings that transient expression of COUP-TF in COUP-TF-negative cells (Fig. 1*B*) restored the ability of *trans*-RA to inhibit TPA-induced AP-1 transactivation and that stable expression of COUP-TF in MBA-MB231 cells enabled *trans*-RA to inhibit TPA-induced c-Jun expression (Fig. 6).

We have reported that COUP-TF expression contributes to growth inhibition by *trans*-RA in cancer cells because COUP-TF on binding to the RARβ promoter induces RARβ expression (35). RARβ is reported to be a potent AP-1 inhibitor

(24), suggesting that COUP-TF-induced RARβ probably contributes to the inhibition of AP-1 activity (Fig. 6). Our observations that COUP-TF can effectively interact with c-Jun *in vitro* (Figs. 3 and 4) and inhibit AP-1 transcriptional activity on transient transfection (Fig. 2) also suggest that COUP-TF is directly involved in antagonizing AP-1 activity.

Inhibition of AP-1 activity appears to be a common characteristic of nuclear receptors, as has been demonstrated for the progesterone, estrogen, androgen, thyroid hormone, glucocorticoid, and retinoid receptors, which can functionally interact with the AP-1 complex. This study reveals that the orphan receptor COUP-TF behaves similarly. The molecular basis of the interaction between nuclear receptors and AP-1 pathways remains largely unknown. Models proposed include direct protein-protein interaction (8, 9, 13–15), inhibition of Jun N-terminal kinase activity (31), and competition for CBP (32). Our results of GST pull-down assays (Figs. 4*B* and 5) and mutation analysis (Figs. 4 and 5) support the first model. The requirement for the COUP-TF DBD for c-Jun binding is similar to observations that the DBDs of glucocorticoid receptor (14, 15) and RAR (47) are essential for their interaction with AP-1. Interestingly, the *in vitro* DNA-binding study showed that COUP-TF is a more effective inhibitor of AP-1 binding than RARα (Fig. 3) on the basis of the levels of each receptor to exert this effect.

In contrast to inhibition of AP-1 activity by other receptors that require their respective ligands, COUP-TF effectively inhibited AP-1 transactivation in the absence of any ligand (Fig. 2). Such a ligand-independent inhibition of AP-1 activity may restrict expression levels of AP-1-responsive genes in cancer cells (Figs. 6 and 7). Interestingly, we observed that COUP-TF expression potentiates antagonism of AP-1 activity by *trans*-RA. However, regulation of *trans*-RA activity by COUP-TF appears to require RARα or RARβ, because transiently transfected COUP-TF was unable to confer anti-AP-1 activity to *trans*-RA in HT-1376 cells lacking RARα or -β (Fig. 8*A*) or HeLa cells (Fig. 8*B*) unless RARα was cotransfected. In addition, stable expression of COUP-TF restored *trans*-RA-induced anti-AP-1 activity in MDA-MB231 cells (Fig. 6) having RARs (48) but not in HT-1376 cells lacking RARα and -β (Fig. 7). Thus, expression of both RARα/β and COUP-TF is required for optimal inhibition of AP-1 activity by *trans*-RA.

Although we do not fully understand how COUP-TF and RARα coexpression maximizes inhibition of AP-1 activity by *trans*-RA, our finding of physical interaction between COUP-TF and RARα (35, 50) suggests its involvement in this process. Based on our observation that COUP-TF more effectively inhibits c-Jun binding to DNA than RARα (Fig. 3), it is tempting to speculate that COUP-TF, with its ability to interact with both RAR (35, 50) and c-Jun (Fig. 3), may function as a bridging factor to mediate RAR/AP-1 interaction. In this model, the COUP-TF/RAR heterodimer would strongly interact with AP-1 in the presence of *trans*-RA to inhibit the DNA binding of AP-1 and its transactivation. Similarly, COUP-TF, by interacting with c-Jun, might also potentiate the inhibition of RARα/β transactivation by AP-1. Unfortunately, perhaps due to rapid formation of the COUP-TF homodimer after COUP-TF expression *in vitro* (40, 51), we were unsuccessful in demonstrating the interaction between RAR/COUP-TF heterodimer and c-Jun in gel shift or GST pull-down assays (data not shown). Another possible mechanism for inhibiting AP-1 transactivation by COUP-TF may involve its recruiting of a transcriptional corepressor. COUP-TF has been widely considered as a potent negative transcriptional regulator (40–43) due to its effective interaction with corepressors, such as N-CoR and SMRT (52). The interaction between COUP-TF and both RAR and AP-1 may recruit transcriptional corepressors to the complex, thereby enhancing the mutual repression of AP-1 and RAR transcriptional activity. Interestingly, COUP-TF interacts with a variety of nuclear receptors, including RXR (41), TR (40, 50), estrogen receptor (53, 54), and peroxisome proliferator-activated receptor (55). It remains to be determined whether and how COUP-TF is involved in modulating the anti-AP-1 activity by their ligands.

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Regulation of Retinoic Acid Receptor β Expression by Peroxisome Proliferator-activated Receptor γ Ligands in Cancer Cells[1]

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ABSTRACT

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor family member that can form a heterodimeric complex with retinoid X receptor (RXR) and initiate transcription of target genes. In this study, we have examined the effects of the PPARγ ligand ciglitazone and the RXR ligand SR11237 on growth and induction of retinoic acid receptor (RAR) β expression in breast and lung cancer cells. Our results demonstrated that ciglitazone and SR11237 cooperatively inhibited the growth of ZR-75-1 and T-47D breast cancer and Calu-6 lung cancer cells. Gel shift analysis indicated that PPARγ, in the presence of RXR, formed a strong complex with a retinoic acid response element (β retinoic acid response element) in the RARβ promoter. In reporter gene assays, RXR ligands and ciglitazone, but not the PPARγ ligand 15d-PGJ_2, cooperatively promoted the transcriptional activity of the β retinoic acid response element. Ciglitazone, but not 15d-PGJ_2, strongly induced RARβ expression in human breast and lung cancer cell lines when used together with SR11237. The induction of RARβ expression by the ciglitazone and SR11237 combination was diminished by a PPARγ-selective antagonist, bisphenol A diglycidyl ether. All-*trans*-retinoic acid or the combination of ciglitazone and SR11237 was able to induce RARβ in all-*trans*-retinoic acid-resistant MDA-MB-231 breast cancer cells only when the orphan receptor chick ovalbumin upstream promoter transcription factor was expressed, or in the presence of the histone deacetylase inhibitor trichostatin A. These studies indicate the existence of a novel RARβ-mediated signaling pathway of PPARγ action, which may provide a molecular basis for developing novel therapies involving RXR and PPARγ ligands in potentiating antitumor responses.

INTRODUCTION

PPARγ[3] is a ligand-activated transcription factor belonging to the steroid/thyroid receptor superfamily, which plays a critical role in the control of adipogenesis (1–4). Specific ligands of PPARγ, including the thiazolidinedione class of antidiabetic agents, the prostanoid 15d-PGJ_2, and certain polyunsaturated fatty acids, have been identified (5, 6). PPARγ expression is not limited to adipocytes because activation of PPARγ by its ligands has been shown to promote growth inhibition, differentiation, and/or apoptosis of various cancer cells (5, 7–9), including breast (10–18) and non-small cell lung carcinoma tissues (19–22).

Retinoids, comprising the native and synthetic derivatives of vitamin A, are promising agents for the prevention and treatment of human cancers, including those of breast and lung (23, 24). The biological effects of retinoids are mainly mediated by their nuclear receptors, RAR and RXR, which each exist as α, β, and γ isoforms (1, 25, 26). PPARγ heterodimerizes with RXR, and the resulting heterodimer binds strongly to its DNA-specific sequence, the PPRE (3, 4). Recent studies have shown that PPARγ/RXR also binds to the estrogen response element (27). PPARγ and RXR ligands have been found to cooperatively induce differentiation and apoptosis of breast and colon cancer cells through interaction with PPARγ/RXR (17, 18, 23, 28).

RARβ plays a critical role in mediating the growth-inhibitory effects of retinoids in various cancer cells (29, 30). The aberrant expression or loss of RARβ in a variety of cancer cell lines suggests that decreased RARβ expression may contribute to retinoid resistance (31–38), implying that RARβ may act as a tumor suppressor. Regulation of RARβ gene expression is principally mediated by the retinoic acid response element (βRARE) in its promoter, to which RAR/RXR heterodimers bind strongly. Activation of RAR/RXR heterodimers is principally mediated via RAR, whereas RXR serves as a silent partner (25). The TR3/RXR heterodimer also binds to the βRARE, which is activated by RXR ligands (39). Thus, the βRARE can be transcriptionally controlled by various heterodimers and their ligands. Other factors also influence RARβ gene expression. The COUP-TF, which is not expressed in many cancer cells, is required for induction of RARβ by ATRA (40). Lack of RARβ expression in cancer cells has also been attributed to abnormal regulation of histone acetylation/deacetylation, which modulates chromatin structure and gene transcription, or hypermethylation of the RARβ promoter (41–45).

In the present study, we evaluated the growth-inhibitory effect of the PPARγ ligand ciglitazone alone and in combination with the RXR ligand (rexinoid) SR11237. Our results showed that ciglitazone and SR11237 cooperatively inhibited the growth and induced apoptosis of breast and lung cancer cell lines. In studying the possible underlying molecular mechanisms, we observed that PPARγ could bind strongly to the βRARE as a PPARγ/RXR heterodimer. The combination of RXR ligands with ciglitazone, but not 15d-PGJ_2, strongly activated the βRARE and induced RARβ expression in breast and lung cancer cells. The induction of RARβ expression by rexinoids and ciglitazone was reduced by a PPARγ antagonist, BADGE (46), indicating the involvement of PPARγ/RXR heterodimers. Together, our results demonstrate that PPARγ can bind to the βRARE as a PPARγ/RXR heterodimer and that induction of RARβ may contribute to the anticancer effect of certain PPARγ ligands.

MATERIALS AND METHODS

Reagents and Cell Lines. The RXR-selective retinoids SR11237, SR11246, and SR11345 were prepared as reported previously (47–49). ATRA and TSA were obtained from Sigma Chemicals (St. Louis, MO). The PPARγ ligands 15d-PGJ_2, ciglitazone, and BADGE were obtained from Cayman Chemicals (Ann Arbor, MI). All reagents were dissolved in a 1:1 ratio of ethanol and DMSO and stored in amber containers at −20°C. Other analytical-grade reagents were obtained from Sigma Chemicals unless otherwise stated.

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[3] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2; ATRA, all-*trans*-retinoic acid; βRARE, β retinoic acid response element; BADGE, bisphenol A diglycidyl ether; COUP-TF, chick ovalbumin upstream promoter transcription factor; DR, direct repeat; HDAC, histone deacetylase; PPRE, peroxisome proliferator response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; TSA, trichostatin A; CAT, chloramphenicol transferase; β-gal, β-galactosidase; MTS, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; BrdUrd, 5-bromo-2′-deoxyuridine; tk, thymidine kinase.

MDA-MB-231, T-47D, and ZR-75-1 cell lines were routinely maintained in DMEM (MDA-MB-231) or RPMI 1640 (T-47D and ZR-75-1), supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). Calu-6 were maintained in MEM Earle's Salt Medium (Irvine Scientific) supplemented with 10% FCS and antibiotics. For for Western analysis studies, cells were cultured in their respective medium supplemented with 5% charcoal-treated FCS and antibiotics.

Plasmids. The PPARγ expression vector was kindly provided by Dr. Mark Leid (Oregon State University, Corvallis, OR). Expression vectors for RXRα and reporter gene βRARE-tk-CAT have been described previously (26, 50).

Transfection Assays. MDA-MB-231 and ZR-75-1 cells were seeded at 2 × 10^5 cells/ml in 6-well plates for 16–24 h before transfection. Cells were transfected with βRARE-tk-CAT plasmid (200 ng), β-gal (200 ng) expression vector (pCH 110; Amersham Biosciences), and carrier DNA (pBluescript; Stratagene, La Jolla, CA) to a final concentration of 2000 ng total DNA/well using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA). Cells were treated for 20 h with RXR and PPARγ ligands. CAT activity was normalized with β-gal activity for transfection efficiency.

Western Blot Analysis. Treated cell cultures were subjected to Western blot analysis as described previously (51). Preblocked nitrocellulose membranes were incubated with 1 μg/ml equivalent of anti-RARβ rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). RARβ protein was detected by horseradish peroxidase-conjugated antirabbit secondary antibody (Amersham Biosciences), and specific bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences). Equivalent loading of samples was determined by reprobing the nitrocellulose membrane with a mouse monoclonal antibody recognizing β-actin (Sigma Chemicals).

Gel Shift Analysis. *In vitro*-synthesized RXR and PPARγ receptor proteins (Promega, Madison, WI) were incubated with RXR and PPARγ ligands alone or in combination and treated with a rabbit polyclonal anti-PPARγ antibody (Santa Cruz Biotechnology) for 20 min before the addition of ^{32}P-labeled βRARE oligonucleotide. Gel shift analysis was performed as described previously (35).

Cell Proliferation and Apoptosis Studies. Treated cells were trypsinized, pelleted by centrifugation at 2000 rpm for 5 min, resuspended in 1 ml of PBS, and fixed in 70% ice-cold ethanol. After two additional PBS washes, the cells were resuspended in PBS containing 50 μg/ml propidium iodide (Sigma Chemicals) and 100 μg/ml DNase-free RNase A (Roche Diagnostics, Indianapolis, IN). Cell suspensions were incubated for 30 min at 37°C with protection from light and analyzed using a FACScatter-Plus flow cytometer (Becton Dickinson, San Jose, CA).

To assess cell viability, cells were seeded at 1 × 10^3 cells/well in 96-well microtiter plates and treated with varying concentrations of SR11237 and ciglitazone, with medium and ligands replaced every 48 h. After treatment, 20 μl of MTS/phenozine methosulfate solution (Promega) were added to each well, and incubation was continued for 2–4 h at 37°C in the dark. Absorbance (490 nm) was measured on a Bio-Rad 550 microplate reader.

BrdUrd Analysis. Treated cells were incubated with BrdUrd (20 μM; Sigma Chemicals) for 2 h before harvesting of cells. After trypsinization and two PBS washes, cells were pelleted by centrifugation and permeabilized with 4% paraformaldehyde. After a 20-min incubation at room temperature, 0.1% saponin (Sigma Chemicals) was added to the cell suspension, and the incubation was continued for 10 min. The cells were then centrifuged, washed twice with PBS containing 0.1% saponin, and resuspended in PBS containing 30 μg of DNase I (Roche Diagnostics). After a 1-h incubation with either an anti-BrdUrd fluorescent antibody or an isotope control antibody (Becton Dickinson), cells were given a final PBS wash before being analyzed using the FACScatter-Plus flow cytometer (Becton Dickinson).

Statistical Analysis. One-way ANOVA with the Dunnett's post test (GraphPad Prism software) was used to assess significance of treatments at the 5% level for growth inhibition studies.

RESULTS

Ciglitazone and SR11237 Cooperatively Inhibit the Growth of Cancer Cell Lines. Both retinoids and PPARγ ligands are potent regulators of cancer cell growth. Because PPARγ heterodimerizes with RXR (5, 52), we investigated the inhibitory effect of their ligands, alone or in combination, on the growth of breast and lung cancer cells. Fig. 1*A* illustrates the effects of the rexinoid SR11237 and the PPARγ ligand ciglitazone on the growth of the hormone-dependent breast cancer cell lines ZR-75-1 and T-47D and the lung cancer cell line Calu-6. Cell proliferation was assessed by MTS assay after 8 (ZR-75-1 and Calu-6) or 10 (T-47D) days of treatment. All three cell lines have been reported to be sensitive to growth inhibition by ATRA (35, 53). Treatment of these cell lines with ATRA strongly inhibited their growth in a dose-dependent manner (Fig. 1*A*). SR11237 did not exhibit a significant effect on T-47D cell growth, whereas in ZR-75-1 and Calu-6 cells, the rexinoid modestly inhibited cell growth when used at 1 μM (10% and 22% for ZR-75-1 and Calu-6 cells, respectively; Fig. 1*A*). Ciglitazone (1 μM) did not have any marked antiproliferative effects in these cancer cell lines. However, when SR11237 and ciglitazone were used in combination, cooperative effects on cell growth were observed in the three cell lines. In ZR-75-1 cells, a 2-fold growth-inhibitory effect was observed when using 1 μM SR11237 and 1 μM ciglitazone (10% with SR11237 *versus* 23% with the combination). In addition, cotreatment of ZR-75-1 cells with 1 μM SR11237 and 10 μM ciglitazone further enhanced growth inhibition by 47% (Fig. 1*A*, *middle graph*). Cotreatment of T-47D cells with SR11237 and ciglitazone resulted in 51% growth inhibition, compared with the antiproliferative effects of each ligand used alone, whereas an additive effect was observed in Calu-6 cells.

We also evaluated the antiproliferative effects of SR11237 and ciglitazone by BrdUrd incorporation. ZR-75-1 cells were treated for 72 h with SR11237 alone or in combination with ciglitazone. As shown in Fig. 1*B*, SR11237 or ciglitazone alone did not inhibit BrdUrd incorporation relative to control. In contrast, the combination decreased BrdUrd incorporation by 46%. Additional studies were performed to determine whether the combination induced apoptosis (Fig. 1*C*). ZR-75-1 cells were treated for 5 days with SR11237 alone or in the presence of ciglitazone. After treatment, cells were harvested and stained with propidium iodide for flow cytometry. Apoptotic cells were determined by measuring sub-G_1-phase cells. When the cells were treated with the combination of SR11237 and ciglitazone, 7.6% of the cells were apoptotic compared with control (Fig. 1*C*). Taken together, these results demonstrate that ciglitazone and a RXR ligand can cooperatively inhibit growth and induce apoptosis in cancer cells.

Induction of RARβ by a PPARγ Ligand in Cancer Cell Lines. Induction of RARβ has been correlated with the growth-inhibitory and apoptosis-inducing effects of retinoids in breast and lung cancer cells (30, 35, 36). We then determined whether the antiproliferative effects observed using rexinoids and PPARγ ligands could be attributed to their ability to induce RARβ. Western analysis was used to investigate whether RXR and PPARγ ligands were capable of regulating RARβ protein expression. ZR-75-1, T-47D, and Calu-6 cells were treated for 24 h with SR11237, in the absence or presence of ciglitazone. In these cell lines, ATRA readily induced RARβ, as reported previously (35), whereas ciglitazone did not show any effect (Fig. 2). SR11237 only weakly induced RARβ in all of the cell lines. However, cotreatment with SR11237 and ciglitazone resulted in a marked expression of RARβ protein. Together, these data demonstrate that ciglitazone strongly induces RARβ expression in combination with a RXR ligand.

Differential Effects of PPARγ Ligands on RARβ Expression. Next, we examined whether other known PPARγ ligands were able to induce RARβ. We evaluated the prostanoid 15d-PGJ_2 and rosiglitazone, two well-characterized PPARγ ligands (5, 6). T-47D, ZR-75-1, and Calu-6 cells were first treated with 15d-PGJ_2 alone or with SR11237. Unlike SR11237 and ciglitazone cotreatment (Fig. 2), SR11237 and 15d-PGJ_2 did not induce RARβ (Fig. 3), thus highlight-

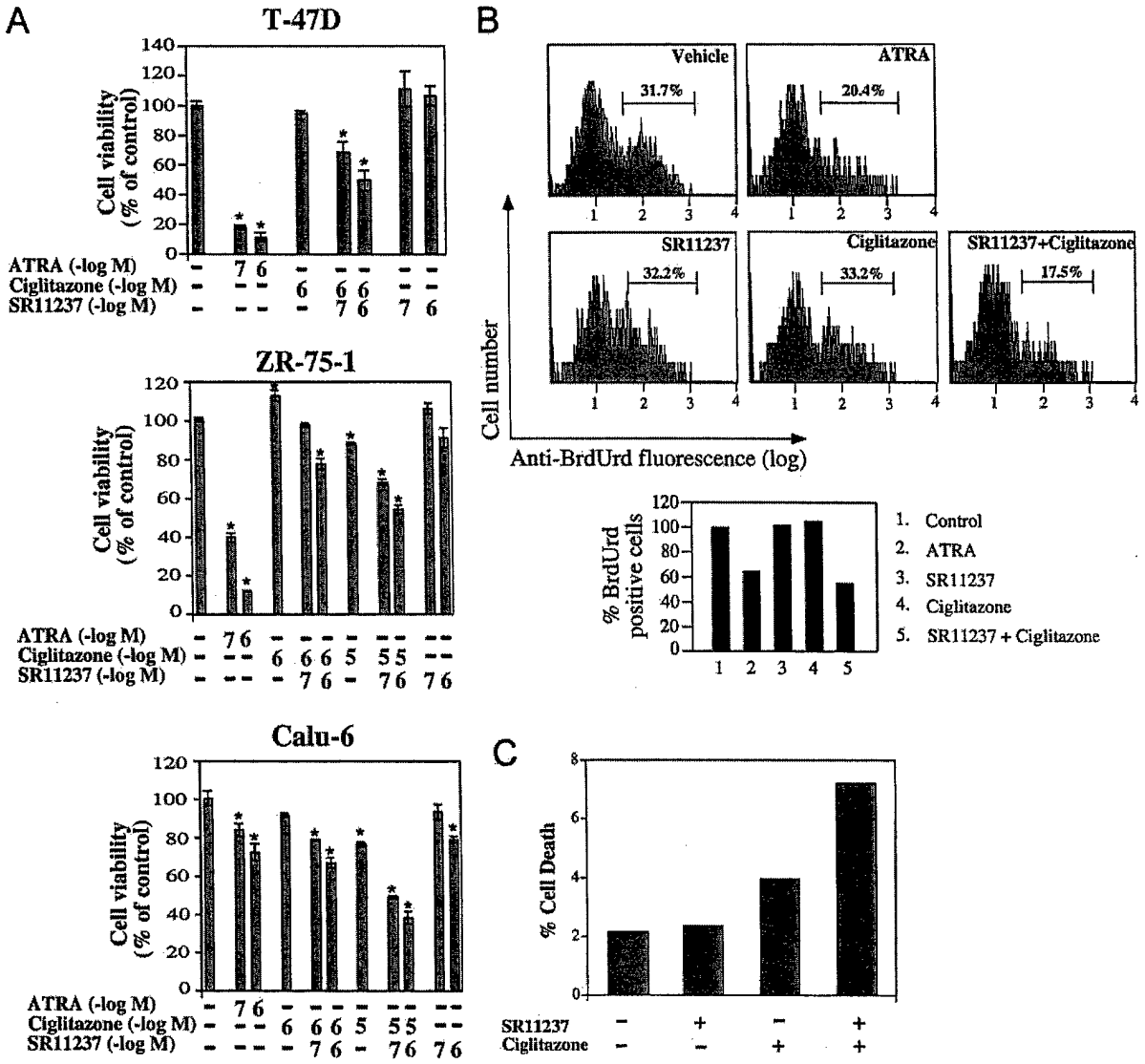

Fig. 1. PPARγ ligand ciglitazone and RXR ligand SR11237 cooperatively inhibit growth and induce apoptosis of cancer cells. *A*, inhibition of cell proliferation by RXR and PPARγ ligands. T-47D, ZR-75-1, and Calu-6 cells were treated for 10 (T-47D) or 8 (ZR-75-1 and Calu-6) days with the indicated concentrations of ATRA, SR11237, or ciglitazone. After treatment, inhibition of cell proliferation was assessed by MTS assay. Cell viability is expressed as a percentage of the control ± SE ($n = 4$). *, $P < 0.05$. *B*, rexinoid and ciglitazone cooperatively inhibit breast cancer cell proliferation. ZR-75-1 cells were treated for 72 h with ATRA alone (1 μM, as positive control) or with SR11237 (1 μM) or ciglitazone (10 μM), either alone or in combination. Cell proliferation was assessed by BrdUrd incorporation (*top graphs*). The data are presented as a histogram (*bottom graph*). *C*, induction of apoptosis by RXR and PPARγ ligands. ZR-75-1 cells were treated for 5 days with SR11237 (1 μM) or ciglitazone (10 μM), either alone or in combination. Apoptotic cells were determined by measuring the sub-G_1 peak of cells using propidium iodide staining and flow cytometry. Results are representative of three separate experiments.

ing the differential effects of the PPARγ ligands on modulating RARβ expression in combination with a RXR-selective retinoid. We also found that rosiglitazone alone or with SR11237 did not induce RARβ (Fig. 3). Thus, not all PPARγ ligands are capable of inducing RARβ expression in cancer cells.

PPARγ Mediates the Effects of Ciglitazone in Inducing RARβ. To determine whether the induction of RARβ by ciglitazone (in combination with SR11237) was mediated by PPARγ, we examined the effect of BADGE a PPARγ antagonist. BADGE was shown to block the ability of PPARγ ligands to activate the transcriptional and adipogenic function of PPARγ (46). Fig. 4 illustrates that BADGE alone did not affect basal RARβ expression in T-47D cells. However, BADGE significantly attenuated ciglitazone/SR11237-induced RARβ. The effect of BADGE was specific because it did not inhibit ATRA-induced RARβ expression. Similar attenuation of ciglitazone/SR11237-induced RARβ by BADGE was observed in ZR-75-1 cells (data not shown). These data strongly suggest that induction of RARβ expression by ciglitazone (in combination with SR11237) is mediated via activation of PPARγ.

PPARγ Binds to the βRARE as a PPARγ/RXR Heterodimer. The βRARE present in the RARβ promoter mediates transcriptional regulation of RARβ expression by RAR and RXR heterodimers (50, 54). To determine whether induction of RARβ by ciglitazone and SR11237 was also mediated via βRARE, we studied the possibility that PPARγ was capable of interacting with the βRARE. *In vitro*-translated PPARγ protein was analyzed for binding to the βRARE by the gel shift assay (Fig. 5*A*). PPARγ alone did not exhibit clear binding to the βRARE. However, PPARγ in combination with *in vitro*-synthesized RXRα protein produced a prominent complex. Binding was not affected when PPARγ/RXR was preincubated with SR11237 or ciglitazone. However, when *in vitro*-translated RXR and PPARγ were incubated with an anti-PPARγ antibody, binding of the RXR/PPARγ heterodimer to βRARE was attenuated, indicating that the PPARγ antibody blocked the ability of PPARγ to bind to βRARE

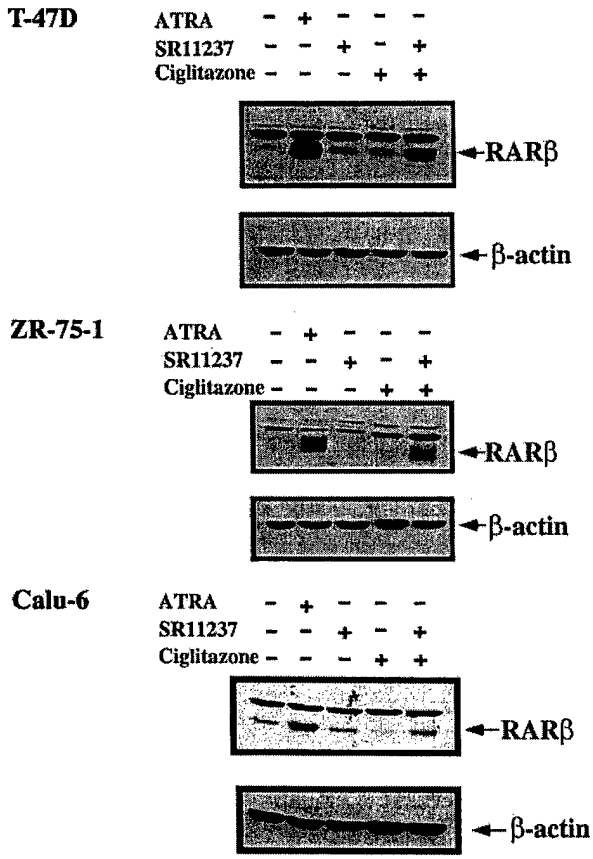

Fig. 2. Regulation of RARβ protein expression by RXR and PPARγ ligands. T-47D, ZR-75-1, and Calu-6 cells were treated for 24 h with SR11237 (1 μM) or ciglitazone (10 μM), either alone or in combination. Cells were also treated with ATRA (1 μM) as a positive control. Cell lysates were prepared, and RARβ protein expression was assessed by Western analysis (51).

(Fig. 5*B*). These data demonstrate that PPARγ can bind to the βRARE as a PPARγ/RXR heterodimer.

Differential Regulation of βRARE Transcriptional Activity by PPARγ Ligands. The observation that PPARγ/RXR binds to the βRARE suggested that it represented another RXR-containing heterodimeric complex that activates the βRARE. Therefore, we studied whether RXR and PPARγ ligands could activate the βRARE. A reporter containing the CAT gene fused to the βRARE (50) was transiently transfected into MDA-MB-231 cells, which were treated with SR11237 alone or in combination with ciglitazone. As revealed in Fig. 6*A*, SR11237, but not ciglitazone, slightly induced βRARE transcriptional activity. Moreover, induction of βRARE was synergized by cotreatment with ciglitazone. We also examined whether the synergistic effect on activating βRARE could be extended to other PPARγ and RXR ligands. Ciglitazone, but not 15d-PGJ_2 or rosiglitazone, strongly induced βRARE activity together with SR11237 (Fig. 6*A*). This correlated with the effects of these PPARγ ligands on induction of RARβ protein expression (Figs. 2 and 3). Similar results were obtained using ZR-75-1 breast cancer cells (Fig. 6*B, top graph*). Thus, not all PPARγ ligands that activate the PPRE similarly activate βRARE together with SR11237. We also examined the effect of the RXR ligands SR11246 and SR11345 on βRARE activity in ZR-75-1 cells (48, 49). Again, ciglitazone strikingly enhanced transcriptional activity of the βRARE induced by SR11246 and SR11345 (Fig. 6*B*).

Antagonistic Effects of Other PPARγ Ligands on RARβ Expression. The differential effect of PPARγ ligands on SR11237-induced RARβ expression (Figs. 2 and 3) prompted us to investigate whether PPARγ ligands antagonized one another's activity. The observation that ciglitazone synergized with SR11237 in inducing RARβ expression suggested that binding of ciglitazone to PPARγ and binding of SR11237 to RXR resulted in transactivation of the βRARE by PPARγ/RXR heterodimers. In contrast, binding of PPARγ with 15d-PGJ_2 may either not activate or suppress the transcriptional activity of PPARγ/RXR on the βRARE. Therefore, we determined whether 15d-PGJ_2 interfered with PPARγ activation by ciglitazone. Fig. 7 shows that in ZR-75-1 cells, 15d-PGJ_2 inhibited RARβ expression induced by the combination of SR11237 and ciglitazone. Thus, 15d-PGJ_2 acts as an antagonist of PPARγ with respect to the induction of RARβ by ciglitazone.

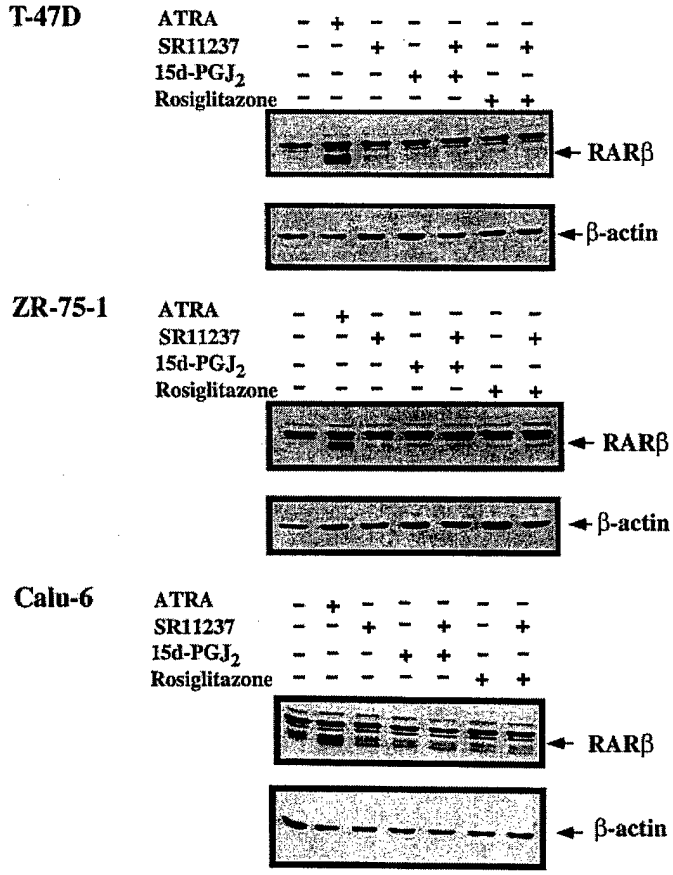

Fig. 3. Differential effects of PPARγ ligands in inducing RARβ expression. T47D, ZR-75-1 and Calu-6 cells were treated for 24 h with SR11237 (1 μM), 15d-PGJ_2 (5 μM), or rosiglitazone (10 μM), alone or with SR11237 + 15d-PGJ_2 or SR11237 + rosiglitazone in combination. Cells were also treated with ATRA (1 μM), as a positive control. RARβ protein expression was assessed by Western analysis.

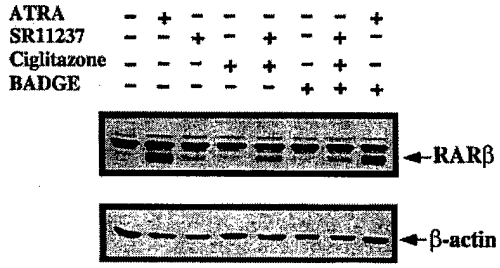

Fig. 4. A PPARγ antagonist blocks induction of RARβ protein expression. T-47D cells were pretreated for 3 h with the PPARγ antagonist BADGE (20 μM), after which the cells were treated with SR11237 (1 μM) or ciglitazone (10 μM), alone or in combination. RARβ protein expression was determined by Western analysis.

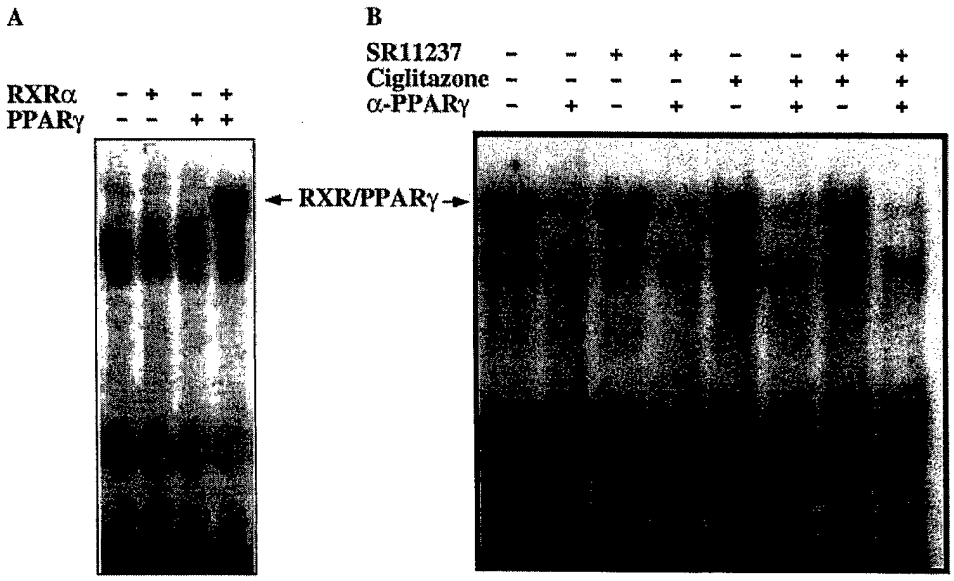

Fig. 5. RXR and PPARγ bind to βRARE as a heterodimeric complex. *A, in vitro*-translated RXR and PPARγ were incubated with ^{32}P-radiolabeled βRARE, either alone or in combination. The resulting reactions were then analyzed by gel shift analysis. *B, in vitro*-translated RXRα and PPARγ were incubated with SR11237 (1 μM) and/or ciglitazone (10 μM), in the absence or presence of anti-PPARγ rabbit polyclonal antibody (α-PPARγ). After a further incubation with ^{32}P-radiolabeled βRARE, the reactions were analyzed by gel shift analysis.

Mechanisms of RARβ Induction in MDA-MB-231 Cells: Role of HDACs and the Orphan Receptor COUP-TF. ATRA does not induce RARβ expression in the estrogen-independent breast cancer cell line MDA-MB-231 (35, 41–43, 55, 56). Therefore, we examined whether SR11237 and ciglitazone could induce RARβ in these cells. In agreement with previous observations (35), treatment of MDA-MB-231 cells with ATRA did not induce RARβ expression (Fig. 8*A*). SR11237 and ciglitazone, either alone or in combination, did not induce RARβ expression, despite the expression of RXRα and PPARγ (data not shown). Histone acetylation and DNA methylation have been reported to contribute to silencing RARβ gene expression in the MDA-MB-231 cell line (41, 44). HDAC inhibitors, such as TSA, have been shown to suppress the effects of corepressors (41). Therefore, to determine whether inhibitors of histone deacetylation played a role in augmenting induction of RARβ by RXR and PPARγ ligands, MDA-MB-231 cells were treated with SR11237, alone or in combination with ciglitazone and TSA. Cotreatment of cells with ATRA and TSA readily induced RARβ protein expression (Fig. 8*A*). Cells cotreated with SR11237, ciglitazone, and TSA also induced RARβ. These results suggest that histone deacetylation may represent a mechanism through which RARβ expression is suppressed in MDA-MB-231 cancer cells.

We reported previously that the orphan receptor COUP-TF is required for RARβ expression by ATRA and is not expressed in MDA-MB-231 cells (40). COUP-TF enhances RARβ expression by increasing the recruitment of receptor coactivators to the RARβ promoter. Stable expression of COUP-TF in MDA-MB-231 cells restored the ability of ATRA to induce RARβ expression (40). Therefore, we examined whether COUP-TF expression was also involved in the induction of RARβ by SR11237 and ciglitazone. Treatment of MDA-MB-231 COUP-TF stable clone cells with ATRA resulted in strong expression of RARβ, as reported previously (40). SR11237 or ciglitazone alone slightly induced RARβ expression in this COUP-TF stable clone (Fig. 8*B*). As observed previously with the T-47D, ZR-75-1, and Calu-6 cells (Fig. 3), 15d-PGJ$_2$, either alone or in combination with SR11237, did not induce RARβ expression in the MDA-MB-231 COUP-TF stable cells. In contrast, SR11237 and ciglitazone markedly induced RARβ expression (Fig. 8*B*). Thus, the expression of COUP-TF modulates the induction of RARβ by PPARγ/RXR heterodimers, as well as the RAR/RXR heterodimers.

DISCUSSION

Retinoids are effective in suppressing tumor development in many animal carcinogenesis models and are being evaluated in clinical trials for prevention and treatment of cancers (23, 24). As an illustration, the rexinoid LGD1069 suppresses mammary tumor development and inhibits the growth of established tumors *in vivo* (57, 58). This retinoid was recently approved for treatment of cutaneous T-cell lymphoma on the basis of clinical trial results and highlights the potential for rexinoids as effective cancer therapeutic agents. Unfortunately, a major limitation in retinoid therapy is that the concentrations needed for anticancer activity also produce adverse effects. Among recent anticancer approaches, combination therapy may lead to synergistic growth-inhibitory effects on cancer cells, thereby allowing the use of lower concentrations to reduce toxicity associated with retinoid treatment (23, 24). In this study, we demonstrate that the PPARγ ligand ciglitazone and the RXR ligand SR11237 cooperatively inhibited the growth of breast and lung cancer cells, whereas either one alone did not markedly inhibit growth (Fig. 1*A*). The combination resulted in an enhanced inhibition of cell growth, BrdUrd incorporation, and induction of apoptosis (Fig. 1). Previous studies showed that the rexinoids enhanced the antidiabetic activity of PPARγ ligands (59). Our present results indicate that the combination of rexinoids and PPARγ ligands may represent a new approach to effectively inhibit the growth of cancer cells.

The cooperative effect of rexinoids and PPARγ ligands is likely mediated by their receptors RXR and PPARγ and their heterodimerization. In elucidation of the downstream pathways mediating the RXR and PPARγ heterodimers, we observed that induction of RARβ, a potent growth inhibitor (29, 30), is involved in the cooperative growth inhibition of PPARγ and RXR ligands. Rexinoids and ciglitazone alone did not show an appreciable effect on RARβ expression. However, their combination strongly induced RARβ expression in breast cancer and lung cancer cells to a degree that was comparable with that of ATRA (Fig. 2). Thus, our results suggest that induction of RARβ expression accounts for the cooperative growth-inhibitory effect of rexinoids and ciglitazone.

The effect of ciglitazone in inducing RARβ is mediated by PPARγ. This was demonstrated by our observation that RARβ induction was attenuated by the PPARγ antagonist BADGE (Fig. 4). Induction of RARβ by ATRA is mainly mediated by RAR/RXR heterodimers that

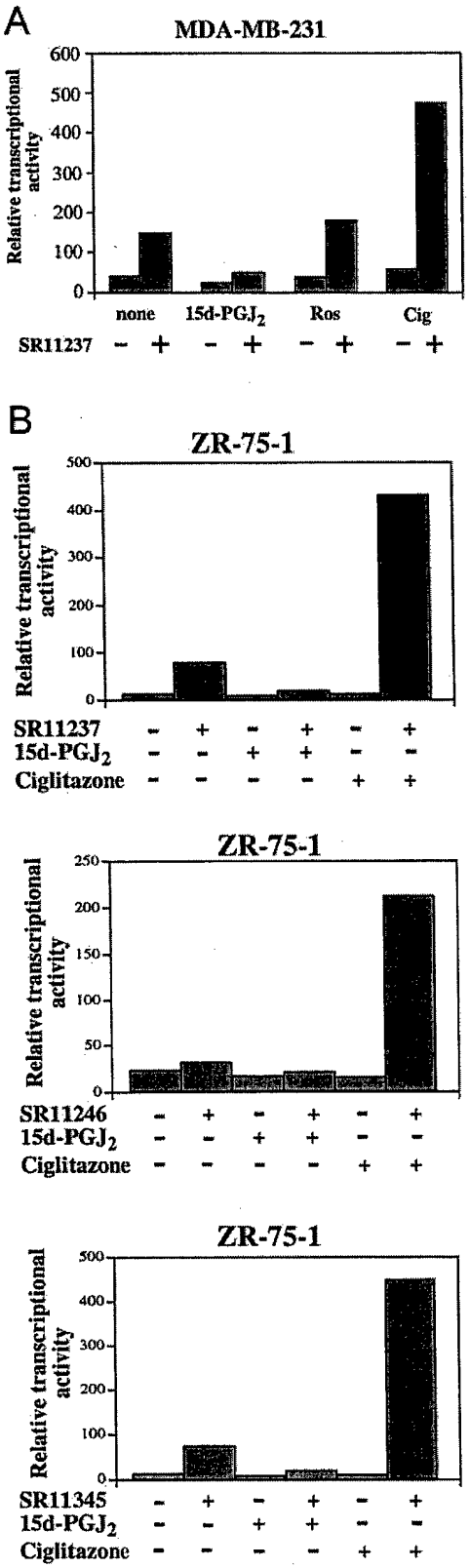

Fig. 6. PPARγ ligands differentially promote βRARE transcriptional activity. The MDA-MB-231 and ZR-75-1 breast cancer cell lines were transiently transfected with 200 ng of βRARE-tk-CAT (50) and 200 ng of β-gal expression vector. Transfected cells were treated for 20 h with the indicated RXR-selective ligands (1 μM) or PPARγ ligands (5 μM 15d-PGJ_2, 10 μM ciglitazone, or rosiglitazone), either alone or in combination. Transcriptional activity of βRARE was assessed by CAT assay, using β-gal as an internal standard to evaluate transfection efficiency. Results are representative of three separate experiments, and values are expressed as relative transcriptional activity. *A*, effect of RXR and PPARγ ligands on inducing βRARE in MDA-MB-231 cells. *B*, effect of RXR and PPARγ ligands on inducing βRARE transcriptional activity in ZR-75-1 cells.

bind to the βRARE in the RARβ promoter (50, 54). In studying how PPARγ and RXR mediated the RARβ induction by rexinoids and ciglitazone, we demonstrated that the PPARγ/RXR heterodimeric complex bound to the βRARE (Fig. 5) and induced its transcriptional activation in the presence of rexinoids and ciglitazone (Fig. 6). We demonstrated previously (39, 60) that rexinoids could induce RARβ expression through TR3/RXR heterodimers via βRARE. The results from the present study demonstrate that the PPARγ/RXR heterodimer represents another RXR-containing heterodimer that mediates the effect of RXR ligands on RARβ induction and growth inhibition.

Classical retinoids fail to induce RARβ expression in certain lung cancer cell lines and in the estrogen-independent MDA-MB-231 breast cancer cells (35, 41–44, 61–64). Lack of RARβ induction has contributed to the retinoid resistance of cancer cells (29, 30). We reported previously (40) that the inability of RAR/RXR heterodimers to activate the RARβ promoter in ATRA-resistant MDA-MB-231 cells was due to lack of the orphan receptor COUP-TF. Our present data indicate that the SR11237 and ciglitazone combination failed to induce RARβ expression in wild-type MDA-MB-231 cells (Fig. 8*A*)

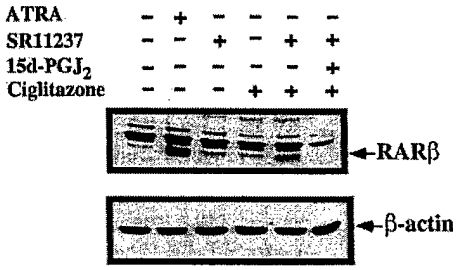

Fig. 7. 15d-PGJ_2 antagonizes RARβ expression induced by rexinoid and ciglitazone. ZR-75-1 cells were treated for 24 h with ATRA alone (1 μM) or with SR11237 (1 μM), ciglitazone (10 μM), or 15d-PGJ_2 (5 μM), either alone or in combination. RARβ protein expression was determined by Western analysis.

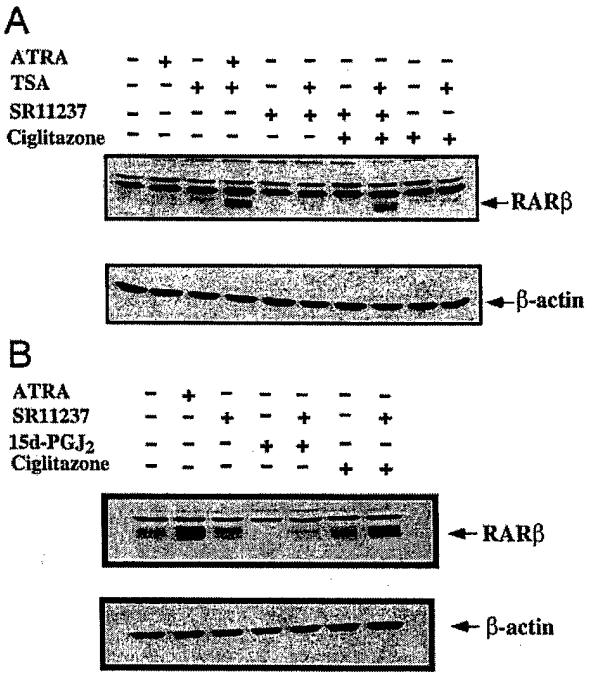

Fig. 8. RXR and PPARγ ligands differentially regulate RARβ expression in wild-type MDA-MB-231 and MDA-MB-231 COUP-TF stable cells. *A*, MDA-MB-231 cells were treated for 48 h with SR11237 (1 μM) ciglitazone (10 μM), or TSA (100 ng/ml), alone or in combination. RARβ protein expression was determined by Western analysis. *B*, MDA-MB-231 COUP-TF stable cells were treated for 24 h with SR11237 (1 μM), 15d-PGJ_2 (5 μM), or ciglitazone (10 μM), alone or in combination, and analyzed for RARβ expression by Western analysis.

but strongly induced RARβ in MDA-MB-231 cells stably expressing COUP-TF (Fig. 8*B*). These results indicate a requirement for COUP-TF in activating RARβ promoter by the PPARγ/RXR heterodimer. Similar to ATRA, we observed that the combination of ciglitazone and SR11237 strongly induced RARβ in wild-type MDA-MB-231 cells when the HDAC inhibitor TSA was present (Fig. 8*A*). These results suggest that histone deacetylation is another mechanism responsible for silencing RARβ expression.

One interesting observation in the present study is that PPARγ ligands differentially regulate βRARE activity and induction of RARβ expression. Ciglitazone, troglitazone, rosiglitazone, and 15d-PGJ_2 act as potent agonists of the PPARγ/RXR heterodimer on the PPRE (3–8). However, only ciglitazone activated βRARE when used with rexinoids (Fig. 6). Combination of rosiglitazone or 15d-PGJ_2 with a rexinoid failed to induce RARβ in these cancer cell lines (Fig. 3). Thus, different PPARγ ligands exhibit opposing effects on transactivation of the PPARγ/RXR heterodimer. Why there is such disparity among the PPARγ compounds and their ability to cooperate with RXR ligands to induce RARβ is presently unclear. One obvious explanation would be the differences in ligand structure, which may bind in an alternate conformation when bound to PPARγ/RXR heterodimers complexed with the βRARE. This difference in binding may then impair or fail to initiate efficient transcription, perhaps through inappropriate recruitment of corepressors or coactivators. Regulation of retinoid signaling by receptor polarity and allosteric control of ligand binding has been well demonstrated for RAR/RXR heterodimers. Binding of RAR/RXR heterodimers with RAR ligand strongly activates the DR5 element, whereas the binding suppresses RXR agonist activity on the DR1 element (65, 66). The differential effects of ligands to activate RAR on response elements was shown to result from opposite polarities of the RXR/RAR heterodimer to asymmetrically oriented half-sites (65). Previous studies demonstrated that PPARγ binds to the 5′-half-site position of the PPRE, whereas RXR occupies the 3′-half site (67, 68). It is likely that similar receptor polarity and allosteric control of transcription of RXR ligand activity by PPARγ ligand binding exists with respect to the PPRE, which is a DR1 response element, and βRARE, a DR5 element. Whether such an allosteric mechanism exists for the PPARγ/RXR heterodimer requires further investigation. Regardless of the underlying molecular mechanisms, our observation provides an opportunity to identify specific PPARγ ligands for inhibiting cancer cell growth through inducing RARβ expression in combination with rexinoids.

In summary, we have demonstrated that rexinoids and ciglitazone can synergistically inhibit the growth of breast and lung cancer cells through their induction of RARβ. Our results demonstrate that PPARγ/RXR heterodimers can bind to the βRARE and promote its transcriptional activity in response to rexinoids and certain PPARγ ligands. Further characterization of the effect of PPARγ ligands on the transactivation of βRARE by PPARγ/RXR heterodimers and the underlying molecular mechanisms may lead to the identification of potent and specific PPARγ ligands that inhibit cancer cell growth through this novel signaling pathway.

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