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Inhibition of *trans*-Retinoic Acid-Resistant Human Breast Cancer Cell Growth by Retinoid X Receptor-Selective Retinoids

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All-*trans*-retinoic acid (*trans*-RA) and other retinoids exert anticancer effects through two types of retinoid receptors, the RA receptors (RARs) and retinoid X receptors (RXRs). Previous studies demonstrated that the growth-inhibitory effects of *trans*-RA and related retinoids are impaired in certain estrogen-independent breast cancer cell lines due to their lower levels of RARα and RARβ. In this study, we evaluated several synthetic retinoids for their ability to induce growth inhibition and apoptosis in both *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cell lines. Our results demonstrate that RXR-selective retinoids, particularly in combination with RAR-selective retinoids, could significantly induce RARβ and inhibit the growth and induce the apoptosis of *trans*-RA-resistant, RARα-deficient MDA-MB-231 cells but had low activity against *trans*-RA-sensitive ZR-75-1 cells that express high levels of RARα. Using gel retardation and transient transfection assays, we found that the effects of RXR-selective retinoids on MDA-MB-231 cells were most likely mediated by RXR-nur77 heterodimers that bound to the RA response element in the RARβ promoter and activated the RARβ promoter in response to RXR-selective retinoids. In contrast, growth inhibition by RAR-selective retinoids in *trans*-RA-sensitive, RARα-expressing cells most probably occurred through RXR-RARα heterodimers that also bound to and activated the RARβ promoter. In MDA-MB-231 clones stably expressing RARα, both RARβ induction and growth inhibition by RXR-selective retinoids were suppressed, while the effects of RAR-selective retinoids were enhanced. Together, our results demonstrate that activation of RXR can inhibit the growth of *trans*-RA-resistant MDA-MB-231 breast cancer cells and suggest that low cellular RARα may regulate the signaling switch from RAR-mediated to RXR-mediated growth inhibition in breast cancer cells.

Retinoids, the natural and synthetic vitamin A analogs, exert profound effects on cell proliferation, differentiation, and apoptosis (19, 36, 50) and are considered promising agents for the prevention and treatment of human cancers, including breast cancer (36, 43, 50). Retinoids, alone or in combination with an antiestrogen or interferons, inhibit the in vitro growth of human breast cancer cells (12–14, 29, 34, 51, 60–62). The natural retinoid derivative retinyl methyl ether (18) and the synthetic retinoids *N*-(4-hydroxyphenyl) retinamide (4-HPR) (44, 45) and LGD1069 (17) effectively inhibited the development of carcinogen-induced mammary cancers in animals. Unfortunately, clinical trials on patients with advanced breast cancer showed no significant activity for retinoids (2, 3, 42). These studies indicate that retinoids are effective inhibitors of the cancer cells at the early stages of tumor progression and that their effectiveness diminishes as cells become more malignant and invasive. They are also consistent with well-documented in vitro observations that growth inhibition by all-*trans*-retinoic acid (*trans*-RA) and related retinoids occurs mainly in estrogen-dependent, estrogen receptor-positive breast cancer cells and that upon progression to estrogen independence and loss of the estrogen receptor, most breast cancer cells become refractory to growth inhibition by *trans*-RA (14, 34, 54, 55, 60).

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (28, 38, 70). 9-*cis*-RA is a high-affinity natural ligand for both RARs and RXRs, whereas *trans*-RA is a high-affinity natural ligand only for the RARs. RARs and RXRs are each encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid hormone/retinoid receptor superfamily, which function as ligand-activated transcription factors (28, 38, 70). RARs interact with RXRs, forming RXR-RAR heterodimers that bind to RA response elements (RAREs) to control the expression of RA-responsive genes in the presence of retinoids. Transcriptional regulation of RA-responsive genes is also modulated by a number of cofactors that appear to provide a direct link to the core transcriptional machinery and/or to modulate chromatin structure (reference 27 and references therein). Although RXR acts as a silent heterodimerization partner of RAR in CV-1 cells (15, 30, 38), recent studies demonstrate that binding of certain RXR ligands contributes to activation of RXR-RAR heterodimers in some cell types (4, 31, 40, 52, 59, 71). In the presence of 9-*cis*-RA, RXRs can also function as homodimers that bind a set of specific DNA sequences (68, 70, 71). Furthermore, activation of RXR is required for the function of other RXR-containing heterodimers, such as RXR-nur77 (15, 49) and RXR-LXR (63). Thus, distinct retinoid signaling pathways through activation of either RAR or RXR exist; however, the role of RXR activation in these pathways requires clarification.

RA target genes, including those for the RARs, have been identified. The RARE (βRARE) in the RARβ gene promoter mediates *trans*-RA-induced RARβ gene expression in many different cell types (10, 21, 57) and binds both RXR-RAR (28, 38, 70) and RXR-nur77 (15, 49) heterodimers. Gene transcriptional activation by RXR-RAR binding is mainly activated by RAR-specific ligands, while transactivation by RXR-nur77 is induced by RXR-specific ligands (15, 49). These observations

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suggest that RARβ can be induced by both RAR- and RXR-specific ligands. nur77 is an orphan member of nuclear receptor superfamily that regulates gene expression by binding to the nur77-binding response element (NBRE) as a monomer (64). It is rapidly induced by various stimuli, including growth factors and phorbol ester- and cyclic AMP-dependent synthesis pathways (20, 39). Recent studies suggest that nur77 is involved in activation-induced apoptosis of T cells (35, 65) and is associated with *trans*-RA resistance in human lung cancer cells (66). Thus, RARβ expression is regulated by growth signals and may be associated with the apoptotic process.

Recently, evidence has emerged that the absence or aberrant expression of RARβ correlates with malignancy and may contribute to the development of cancers. The involvement of RARβ in cancer development was originally implicated in the finding that the RARβ gene is integrated by hepatitis B virus in human liver cancer (9). Subsequent reports indicate that abnormal expression of the RARβ gene appears to be involved in the tumorigenicity of human papillomavirus type 18-transformed ovarian cancer cells (1) and the neoplastic progression of human oral squamous cell carcinoma cell lines (24), and it is observed in many other human cancer cell lines (16, 22, 24, 34, 48, 58, 69). RARβ also suppresses the growth of breast cancer cells (33, 34, 58) and lung cancer cells (23). The autoinduction of RARβ gene expression presumably plays a critical role in amplifying retinoid responses and is associated with the growth-inhibitory effects of *trans*-RA in breast cancer cells in vitro (34) and the clinical response to retinoids in patients with premalignant oral lesions (37). Retinoids that fail to induce RARβ expression cannot arrest the growth of melanoma cells (5).

The involvement of retinoid receptors in mediating retinoid-induced growth inhibition and apoptosis has been investigated. Several studies demonstrated that expression of RARα mediates the growth-inhibitory effect of *trans*-RA in estrogen-dependent breast cancer cells and that the loss of *trans*-RA sensitivity in estrogen-independent cells may be due to low levels of RARα (34, 51, 55, 60). RARα levels are higher in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1, MCF-7, and T-47D, than in certain estrogen-independent, *trans*-RA-resistant cells lines, such as MDA-MB-231 and MDA-MB-468 (34, 51, 55, 60). Expression of RARα in estrogen-independent, *trans*-RA-resistant MDA-MB-231 breast cancer cells restored *trans*-RA sensitivity (34, 54, 55, 61). Growth inhibition induced by retinoids in estrogen-dependent MCF-7 breast cancer cells correlated with their binding affinity to RARα (7). The involvement of RARβ was suggested by the observation that it was expressed in response to *trans*-RA in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1 and T-47D, but not in estrogen-independent, *trans*-RA-resistant cell lines MDA-MB-231, MDA-MB-468, and BT-20 (34). In addition, we (34) and others (33, 53) demonstrated that introduction of RARβ into MDA-MB-231 cells led to the recovery of *trans*-RA-induced growth inhibition. Moreover, RARβ expression was enhanced in RARα stably transfected MDA-MB-231 cells, a finding that suggests that RARβ may mediate the growth inhibitory effects of RARα (34). The role of RARβ in growth inhibition is also supported by the observation that as normal human mammary epithelial cells senesce, RARβ mRNA expression increases (58). RARγ is highly expressed in various breast cancer cell lines independently of their estrogen responsiveness (34) and is unlikely involved in regulating *trans*-RA-induced growth inhibition and apoptosis. However, recent studies (11, 12) have demonstrated that it may play a role in mediating growth inhibition and apoptosis induction by 4-HPR and certain synthetic retinoids. 4-HPR was a potent transactivator of RARγ at concentrations that inhibited the growth and induced apoptosis of breast cancer cells (11). Furthermore, growth inhibition by certain receptor-selective retinoids and interferons was associated with increased expression of RARγ (12). Thus, different retinoid receptors, which may function through different mechanisms, can mediate growth inhibition and apoptosis induction by different types of retinoids in breast cancer cells.

In this study, we evaluated the effects of RAR- and RXR-class selective retinoids on the growth of *trans*-RA-resistant, RARα-deficient MDA-MB-231 cells. Our results demonstrate that RXR-selective retinoids induced RARβ expression, growth inhibition, and apoptosis in these cells, most likely through their activation of RXR-nur77 heterodimers that bind to the RARβ promoter. When we stably expressed RARα in MDA-MB-231 cells, we observed an enhanced growth inhibition and RARβ induction by RAR-selective retinoids and decreased effects by RXR-selective retinoids, similar to those observed in *trans*-RA-sensitive, RARα-expressing breast cancer cells, such as ZR-75-1 cells. Thus, an RXR-mediated growth inhibition pathway exists in breast cancer cells and is regulated by RARα levels. These results may provide a novel method for inhibiting the growth of the more malignant *trans*-RA-resistant breast cancer cells.

MATERIALS AND METHODS

Retinoids. *trans*-RA was obtained from Sigma (St. Louis, Mo.). SR11246, SR11237, and SR11235 were prepared as described by Dawson et al. (8). Synthesis of SR11383 was described elsewhere (16a).

(*E*)-3-[4-(1-Methoxy-5,6,7,8-tetrahydro-1-5,5,8,8-tetramethyl-3-naphthalenyl) phenyl]propenoic acid (SR11278) was synthesized as follows. (i) Cyclialkylation [$AlCl_3$, $(CH_2Cl)_2$, 0°C] of 3-bromoanisole with 2,5-dichloro-2,5-dimethylhexane as reported by Kagechika et al. (26) yielded 3-bromo-1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (71%), which was coupled under Suzuki conditions {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous 1,2-dimethoxyethane [MeO $(CH_2)_2OMe$], reflux} (41) with 4-formylphenylboronic acid to give 4-(1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-3-naphthalenyl)benzaldehyde (91%). (ii) Horner-Emmons olefination of this benzaldehyde with triethyl phosphonoacetate [$KN(SiMe_3)_2$, tetrahydrofuran-toluene, −78 to 25°C] produced the ethyl ester of SR11278 (97%). (iii) Hydrolysis (KOH, aqueous ethanol [EtOH]; aqueous HCl) gave SR11278 (99%): melting point (mp), 179 to 182°C; ^{1}H nuclear magnetic resonance (NMR) (300 MHz, $^2HCCl_3$) δ 1.34 (s, 6, CMe_2), 1.41 (s, 6, CMe_2), 1.68 (m, 4, CH_2CH_2), 3.89 (s, 3, OMe), 6.49 (d, J = 16.0 Hz, 1, $C{=}CHCO_2$), 6.90 (d, J = 1.8 Hz, 1, ArH), 7.20 (d, J = 1.8 Hz, 1, ArH), 7.62 (s, 4, ArH), 7.83 (d, J = 16.0 Hz, 1, $HC{=}CCO_2$); infrared (IR) (KBr) 2,955, 1,700, 1,630, 1,430, 1,278, 1,220, 825 cm^{-1}.

4-[(3-Hydroxy-5,6,7,8-tetrahydro-3,5,5,8,8-tetramethyl-2-naphthalenyl)carboxyamido]benzoic acid (SR11281) was synthesized as follows. (i) Fries rearrangement ($AlCl_3$, 130°C [23]) of 2-acetoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene produced 1-(3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)ethanone (90%). (ii) Protection of the phenolic group as the benzyl ether (benzyl bromide, K_2CO_3, acetone, reflux; 92%) and oxidation of the acetyl group (NaOCl, EtOH, reflux) gave 3-benzyloxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carboxylic acid (30%). (iii) The carboxylic acid was converted (oxalyl chloride, CH_2Cl_2) to the acyl chloride and treated (pyridine-benzene) with ethyl 4-aminobenzoate to yield the benzamide (95%). (iv) Ester hydrolysis (NaOH, aqueous EtOH, 25°C; aqueous HCl; 97%) and hydrogenolysis [H_2, Pd(C), EtOH, 25°C; 93%] of the benzyl ether protecting group afforded SR11281: mp, 275 to 278°C; ^{1}H NMR (300 MHz, $^2HCCl_3$) δ 1.29 (s, 6, CMe_2), 1.32 (s, 6, CMe_3), 1.69 (s, 4, CH_2CH_2), 6.93 (s, 1, ArH), 7.69 (s, 1, ArH), 7.74 (d, 2, J = 8.8 Hz, ArH), 8.08 (d, 2, J = 8.8 Hz, ArH); IR (KBr) 3,330, 1,686, 1,530, 1,419, 1,174 cm^{-1}.

4-[1-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid (SR11345) was prepared by Suzuki Pd(0)-catalyzed coupling {$Pd[P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous $MeO(CH_2)_2OMe$, reflux; 58%} (8) between 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene-2-boronic acid and methyl (1-bromo-2-methylpropenyl)benzoate and hydrolysis (KOH, aqueous EtOH; aqueous HCl; 91%). The first intermediate was synthesized in two steps by bromination (Br_2, $CHCl_3$, 25°C; 80%) of 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene, followed by conversion to the arylboronic acid [*n*-butyllithium (*n*-BuLi), tetrahydrofuran (THF), −78°C to ambient temperature; $B(OMe)_3$, −78°C to ambient temperature; aqueous NH_4Cl; 86%]. The second intermediate was obtained from methyl (2-methylpropenyl)benzoate by bromination (Br_2, CH_2Cl_2; 100%) and dehydrobromination {1,8-diazabicyclo[5.4.0]undec-7-ene, $MeO(CH_2)_2OMe$, 25°C; 90%} to yield SR11345: mp, 246 to 247°C; ^{1}H NMR

($^2HCCl_3$) δ 1.25 (s, 12, $4CH_3$), 1.64 (s, 3, CH_3), 1.66 (s, 3, CH_3), 1.88 [s, 4, $(CH_2)_2$], 1.99 (s, 3, CH_3), 7.00 (s, 1, ArH), 7.03 (s, 1, ArH), 7.25 (d, J = 8.3, 2, ArH), 7.98 (d, J = 8.3, 2, ArH); IR (KBr) 3,500 to 2,300 (OH), 1,687 (C = O), 1,606 (C = C) cm^{-1}; chemical ionization high-resolution mass spectrum (CI-HRMS) (NH_3) calculated for $C_{26}H_{32}O_2 + NH_4^+$, 394.2746; found, 394.2751.

4-(1-Amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethylanthracen-2-yl)benzoic acid (SR11350) was synthesized by nitration (HNO_3, acetic anhydride-acetic acid [Ac_2O-HOAc], −10°C) of 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethylanthracene to give after chromatographic separation (silica, CH_2Cl_2-hexanes) of isomers 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-5-nitroanthracene (26%), which was coupled {Pd[$P(C_6H_5)_3]_4$, $NaHCO_3$, aqueous dimethyl ether (DME), 80°C; 83%} to 4-carbethoxyphenylboronic acid to afford ethyl 4-(1-nitro-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-anthracen-2-yl)benzoate. Hydrogenation [H_2, Pd(C), EtOAc, 25°C; 95%) to the amine and ester hydrolysis (KOH, aqueous EtOH; 85°C; aqueous HCl; 93%) yielded SR11350 as the HCl: salt mp, 267 to 268°C; ^1H NMR (300 MHz, Me_2SO-$^2H_6$) δ 1.40 (s, 6, CH_3), 1.44 (s, 6, CH_3), 1.77 (s, 4, CH_2), 7.25 (d, J = 8.6 Hz, 1, ArH) 7.55 (d, J = 8.6 Hz, 1, ArH), 7.55 (d, J = 8.2 Hz, 2, ArH), 7.90 (s, 1, ArH), 8.11 (d, J = 8.2 Hz, 2, ArH), 8.21 (s, 1, ArH); electron impact high-resolution mass spectrum (EI-HRMS) calculated for $C_{25}H_{27}NO_2$, 373.2042; found, 373.2040.

6-[3-(1-Adamantyl)-5-methoxyphenyl]naphthalene-2-carboxylic acid (SR11362) was obtained by hydrolysis (KOH, aqueous EtOH, 85°C; aqueous HCl; 90%) of its ethyl ester: mp, 236 to 238°C; ^1H NMR (300 MHz, $^2HCCl3$) δ 1.79 (m, 6, CH_2), 1.98 (m, 6, CH_2), 2.12 (m, 3, CH), 3.87 (s, 3, OCH_3), 6.96 (s, 1, ArH), 7.02 (s, 1, ArH), 7.28 (s, 1, ArH), 7.75 (m, 1, ArH), 7.96 (m, 3, ArH), 8.12 (d, J = Hz, 1, ArH), 8.68 (s, 1, ArH); EI-HRMS calculated for $C_{28}H_{28}O_3$, 412.2038; found, 412.2030.

2-(4-Carboxyphenyl)-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[*g*]quinoline-1-oxide (SR11365) was prepared by acetylation (Ac_2O, Et_3N, EtOAc, 25°C; 100%) of 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-amine to the amide, which on reaction with excess Vilsmeier reagent {$[POC]_3$, 8.5 equivalents; alerts dimethylformamide (DMF) 3.5 equivalents; alerts $(CH_2Cl)_2$, 0°C]; 25 to 85°C; aqueous $NaHCO_3$; 61%} produced 2-chloro-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[*g*]quinoline-3-carboxaldehyde, which on coupling {Pd[P$(C_6H_5)_3]_4$, $NaHCO_3$, aqueous DME, 80°C; 71%} with 4-carbethoxyphenylboronic acid yielded ethyl 4-[3-formyl-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo-[g]quinolin-2-yl]benzoate. Oxidation of the formyl group to the carboxylic acid with concomitant base hydrolysis of the ester (Ag_2O, NaOH, aqueous EtOH-THF, 25°C; H_3O^+; 100%) gave the dicarboxylic acid, which on thermal decarboxylation (315°C; 93%) yielded 4-[6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo-(*g*)quinolin-2-yl]benzoic acid, which has also been synthesized by another route (26). Esterification ($SOCl_2$, reflux; MeOH-C_5H_5N-$C_6H_5CH_3$; aqueous $NaHCO_3$; 91%), *N*-oxidation (3-$ClC_6H_4CO_3H$, $CHCl_3$, 25°C; 85%), and ester hydrolysis (NaOH, aqueous EtOH, 70°C; aqueous citric acid; 72%) yielded SR11365: mp, >300°C; ^1H NMR (300 MHz, Me_2SO-$^2H_6$) δ 1.42 (s, 12, CH_3), 1.80 (s, 4, CH_2), 7.68 (d, J = 9 Hz, 1, ArH), 7.95 (d, J = 9 Hz, 1, ArH), 8.10 (d, J = 8 Hz, 2, ArH), 8.14 (s, 1, ArH), 8.15 (d, J = 8 Hz, 2H, ArH), 8.63 (s, 1, ArH); EI-HRMS calculated for $C_{24}H_{25}NO_3$, 375.1834; found, 375.1832.

Cell culture. Breast cancer cell lines ZR-75-1, T-47D, and MDA-MB-231 were obtained from the American Type Culture Collection. ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, and MDA-MB-231 and CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Growth inhibition assay. To study anchorage-dependent growth inhibition, cells were seeded at 500 cells per well in 96-well plates and treated with solvent control (dimethyl sulfoxide-EtOH) or with 10^{-6} M retinoids (or 10^{-7} M *trans*-RA) in solvent. Media were changed every 48 h. After treatment for 10 days, the number of viable cells were determined by their capacity to convert the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) into a blue formazan product, using a cell proliferation-cytotoxicity assay kit (Promega, Madison, Wis.) (46).

RNA preparation and Northern blot. For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (34). About 30-μg aliquots of total RNAs from different cell lines treated with or without 10^{-6} M retinoids (or 10^{-7} M *trans*-RA) were fractionated on 1% agarose gel, transferred to nylon filters, and probed with the ^{32}P-labeled ligand-binding domain of receptor cDNAs as previously described (34). To determine that equal amounts of RNA were used, the filters were also probed with β-actin.

Plasmids, receptor proteins, and nuclear extract preparation. The nur77 expression vector was constructed by cloning the nur77 cDNA fragment into pECE or pBluescript vector as described previously (66). The construction of the chloramphenicol acetyltransferase (CAT) reporter containing the RARβ promoter (*Bgl*II-*Bam*HI fragment) and expression vectors for RARα, RARβ, and RXRα have been described elsewhere (21, 67, 68). The RXRα N-terminal deletion mutant (ΔRXR10) was constructed by deleting 61 amino acid residues from its N-terminal end as described previously (71). Receptor proteins were synthesized by an in vitro transcription-translation system using rabbit reticulocyte lysate (Promega) as described previously (67). The relative amount of the translated proteins was determined by [^{35}S]methionine-labeled protein on sodium dodecyl sulfate-polyacrylamide gels by quantitating and then normalizing the amount of incorporated radioactivity relative to the content of methionine in each protein. Nuclear extracts were prepared as previously described (34).

TABLE 1. Retinoid transcriptional activation activity

Retinoid	Relative receptor transactivation (%)[a]			
	RARα	RARβ	RARγ	RXRα
trans-RA	100	100	100	53
9-*cis*-RA				100
SR11277	5	149	65	0
SR11278	13	145	67	0
SR11281	59	123	133	0
SR11365	95	56	48	6
SR11383	66	71	16	7
SR11235	3	27	0	51
SR11246	6	12	9	98
SR11237	1	44	0	95
SR11345	0	−6	−12	107

[a] Transcriptional activation in CV-1 cells, using the $(TREpal)_2$-*tk*-CAT reporter, compared to 1 μM *trans*-RA for RARs or 1 μM 9-*cis*-RA for RXRα as 100%.

Gel retardation assay. The gel retardation assay using in vitro-synthesized proteins or nuclear extracts has been described previously (67, 68). When antibodies were used, 1 μl of anti-nur77 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) or 1 μl of anti-RXR (32) was incubated with receptor protein at room temperature for 30 min prior to the assay.

Transient transfection and CAT assay. To measure transcriptional activation of the RARβ promoter by retinoids, this promoter (*Bgl*II-*Bam*HI fragment) was linked to the CAT gene reporter (21). The reporter plasmid and β-galactosidase expression vector (pCH110; Pharmacia) (100 ng each) with or without the RARβ expression vector were transiently transfected into CV-1 cells by the calcium phosphate precipitation method (34). Cells were grown in the presence or absence of 10^{-6} M retinoids or 10^{-7} M *trans*-RA. Transfection efficiency was normalized to β-galactosidase activity. The data shown are the means of three separate experiments.

Stable transfection. RARα cDNA was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.) as described elsewhere (34). The resulting recombinant constructs were then stably transfected into MDA-MB-231 breast cancer cells by the calcium phosphate precipitation method and screened by using G418 (Gibco BRL, Grand Island, N.Y.). The levels of exogenous RARα expression were determined by Northern blotting.

Apoptosis analysis. Cells were treated with or without 10^{-6} M retinoids (or 10^{-7} M *trans*-RA). After 48 h, cells were trypsinized, washed with phosphate-buffered saline (PBS; pH 7.4), and fixed in 1% formaldehyde in PBS. After washing with PBS, cells were resuspended in 70% ice-cold EtOH and immediately stored at −20°C overnight. Cells were then labeled with biotin-16-dUTP by terminal deoxynucleotidyltransferase (TdT) and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescently labeled cells were analyzed using a FACScater-Plus as described previously (34). Representative histograms are shown.

RESULTS

RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 but not *trans*-RA-sensitive ZR-75-1 cells. Because *trans*-RA effectively inhibited the growth and induced the apoptosis of *trans*-RA-sensitive, estrogen-dependent ZR-75-1 breast cancer cells, whereas it had little effect on *trans*-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells (34), RAR- and RXR-class selective retinoids (Table 1) were evaluated for the ability to inhibit the growth and induce the apoptosis of these cell lines. At 10^{-6} M, SR11278, SR11281, SR11277, SR11383, and SR11365 activated only the RARs, not RXRα, on the $(TREpal)_2$-*tk*-CAT reporter construct (67, 68), as determined by transient transfection in CV-1 cells. SR11237, SR11246, and SR11235 activated both RXRα and RARβ, whereas SR11345 activated only RXRα (Table 1). Both breast cancer cell lines were treated for 10 days with 10^{-6} M the indicated class-selective retinoid alone or the combination of RXR-selective SR11345 and a RAR-selective retinoid. Cell viability was determined by the MTT assay. As shown in Fig. 1, the RAR-selective retinoids strongly inhibited ZR-75-1 cell growth (55

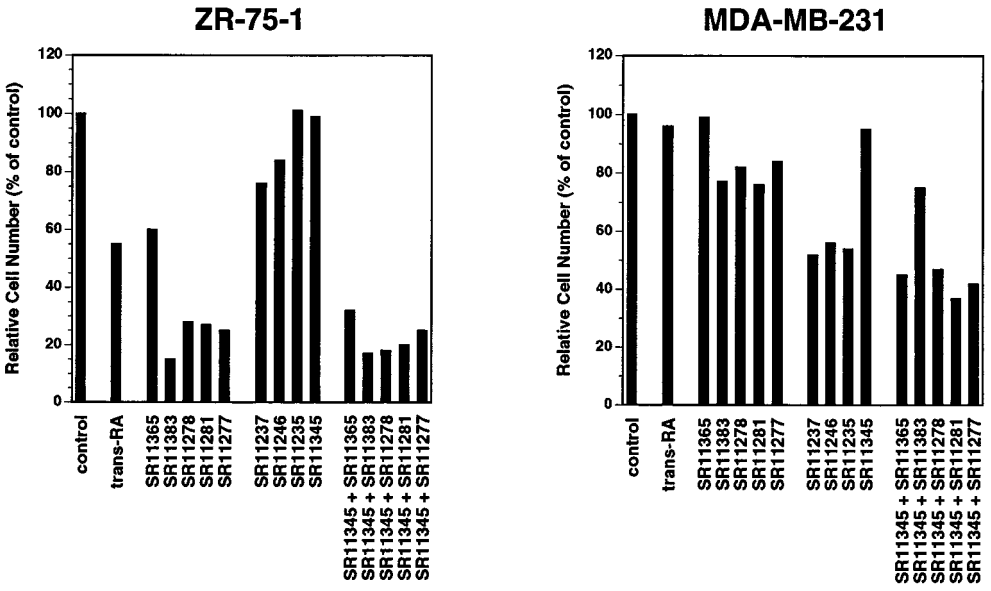

FIG. 1. Growth-inhibitory effects of retinoids on *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and *trans*-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells. Cells (800 cells/well) were seeded in 96-well plates and treated with the indicated retinoids (10^{-6} M) or *trans*-RA (10^{-7} M) alone or in combination for 10 days. The number of viable cells was determined by the MTT assay.

to 75%). In contrast, the RXR-selective retinoids were far less effective inhibitors (8 to 25%) of growth. ZR-75-1 cell growth inhibition by any of the RAR-selective retinoids was only slightly enhanced by the RXR-selective SR11345. Therefore, growth inhibition of *trans*-RA-sensitive ZR-75-1 cells by retinoids is mediated mainly by the RAR pathway, not the RXR pathway, a finding which is consistent with previous observations (34, 55). Interestingly, in MDA-MB-231 cells, RXR-selective SR11237, SR11246, or SR11235 at 10^{-6} M inhibited growth (45 to 50%) more effectively than any RAR-selective retinoid, which was a poor inhibitor (<20%) (Fig. 1). The more RXR-specific retinoid SR11345 was less effective, with only 8% inhibition. However, when it was used together with one of the RAR-selective retinoids, growth inhibition was increased to 40 to 55%. These results indicate that activation of both RAR and RXR signaling pathways is required for effective cancer cell growth inhibition.

We next investigated the apoptosis-inducing effects of RAR-selective SR11365 and *trans*-RA and of RXR-selective SR11246 in ZR-75-1 and MDA-MB-231 cells by the TdT assay (Fig. 2). *trans*-RA at 10^{-7} M and SR11365 at 10^{-6} M significantly induced apoptosis of ZR-75-1 cells, producing 58 and 27% apoptosis, respectively, whereas RXR-selective SR11246 produced only about 5% apoptotic cells (Fig. 2A). About 44% of MDA-MB-231 cells underwent apoptosis on treatment with SR11246, but apoptosis was not significant on treatment with *trans*-RA (4%) or SR11365 (6%) (Fig. 2B). Together, these results demonstrated that RXR-selective retinoids can induce growth inhibition and apoptosis of *trans*-RA-resistant MDA-MB-231 cells, whereas RAR-selective retinoids are more effective in *trans*-RA-sensitive cells.

Induction of RARβ in *trans*-RA-resistant breast cancer cells by RXR-selective retinoids. We previously demonstrated that RARβ expression levels in breast cancer cells correlated with the extent of growth inhibition and apoptosis induction by *trans*-RA (34). To determine whether growth inhibition and apoptosis induction by RXR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells were also associated with their induction of RARβ, we compared the effect of RXR-selective retinoids SR11246 and SR11345 and RAR-selective SR11365, as well as the SR11345 and SR11365 combination, on RARβ expression in these cells (Fig. 3). For comparison, *trans*-RA-sensitive ZR-75-1 and T-47D cells were studied. Both *trans*-RA and RAR-selective SR11365, but not RXR-selective SR11246 or SR11345, induced RARβ expression in ZR-75-1 and T-47D cells. However, RXR-selective SR11246 and SR11345 induced RARβ expression in *trans*-RA-resistant MDA-MB-231 cells at a level comparable to that observed with *trans*-RA or SR11365. A further induction of RARβ was observed when MDA-MB-231 cells were treated with both RXR-selective SR11345 and RAR-selective SR11365. These results demonstrate that activation of RXR by RXR-selective retinoids induced RARβ in *trans*-RA-resistant MDA-MB-231 cells, while these retinoids were unable to activate the RXR-pathway for inducing RARβ in *trans*-RA-sensitive ZR-75-1 and T-47D breast cancer cells. Thus, induction of RARβ by RXR-selective retinoids may contribute to the effects of these retinoids on growth inhibition and apoptosis induction in *trans*-RA-resistant MDA-MB-231 cells.

RXR-selective retinoids activate the RARβ promoter through RXR-nur77 heterodimers. RXR ligands can regulate gene expression through RXR homodimers (68) or certain RXR heterodimers, such as RXR-RAR (4, 31), RXR-LXR (63), or RXR-nur77 (15, 49). Regulation of gene expression by RXR-nur77 heterodimers occurs through their binding to DR-5 type RAREs (15, 49). Because the βRARE in the RARβ promoter is a DR-5 type RARE and contains an NBRE (15, 49, 64), we investigated whether induction of RARβ expression in MDA-MB-231 cells (Fig. 3) could be due to apparent activation of RXR-nur77 heterodimers on the βRARE by RXR-selective retinoids. nur77 alone did not show any clear binding to the βRARE under our experimental conditions but in the presence of RXR produced a strong complex, whose binding was largely affected by either anti-RXR or anti-nur77 antibody (Fig. 4A). For a better distinction between RXR-RAR and RXR-nur77 heterodimers, an RXRα mutant with a deletion of

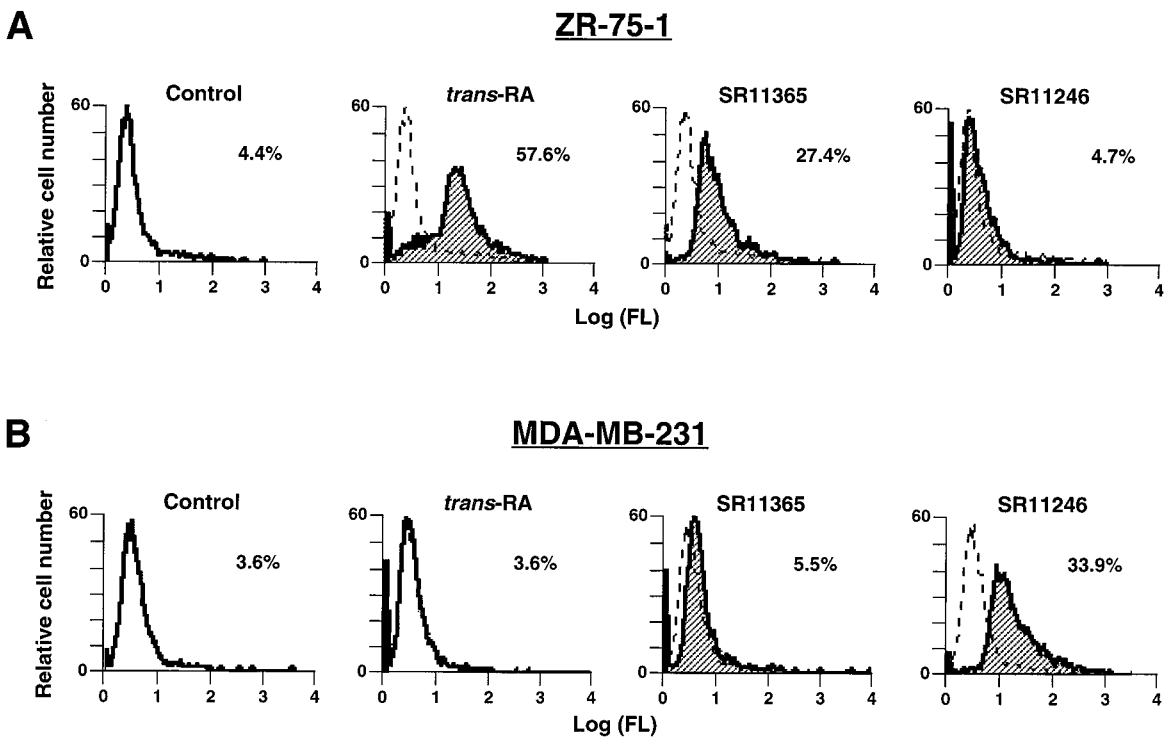

FIG. 2. Induction of apoptosis by retinoids in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 (A) and *trans*-RA-resistant, estrogen-independent MDA-MB-231 (B) breast cancer cells. Breast cancer cells were grown in the presence of the indicated retinoids at 10^{-6} M or *trans*-RA at 10^{-7} M for 48 h. DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell number. FL, fluorescence.

68 amino acid residues from its N-terminal end (71) was used. The deletion did not affect heterodimerization properties of the RXR with RAR or nur77 (data not shown). The binding of the RXR-nur77 heterodimers to the βRARE was comparable to that of the RXR-RAR heterodimers (Fig. 4A). Thus, the unique structure of the βRARE permits binding of both RXR-RAR and RXR-nur77 heterodimers, as previously observed (15, 49). We next carried out transient transfection assays in CV-1 cells, using the RARβ promoter linked to the CAT gene (21) as a reporter. As shown in Fig. 4B, cotransfection of the RXRα expression vector did not induce reporter transcriptional activity in response to RXR-selective SR11246 or SR11345, a result that suggests that RXR homodimers do not activate the RARβ promoter as previously observed (68). Cotransfection of the nur77 expression vector clearly induced reporter activity in response to these RXR-selective retinoids but not to RAR-selective *trans*-RA or SR11365. When nur77 and RXR expression vectors were cotransfected, a stronger induction of reporter activity was observed when cells were treated with the RXR-selective retinoids but not with *trans*-RA or SR11365. To determine the effect of RARα on RXR-nur77 activity, we cotransfected the RARα expression vector together with RXRα and nur77. Addition of RARα strongly inhibited RXR-selective retinoid-induced reporter activity but significantly enhanced *trans*-RA and SR11365 activity (Fig. 4B). The inhibition of SR11246 and SR11345 activity by RARα is likely due to competition of RARα and nur77 for heterodimerization with RXR and binding to the βRARE, which suggests that RXR-selective retinoids SR11246 and SR11345 cannot activate RARβ promoter through RXR-RAR heterodimers in CV-1 cells.

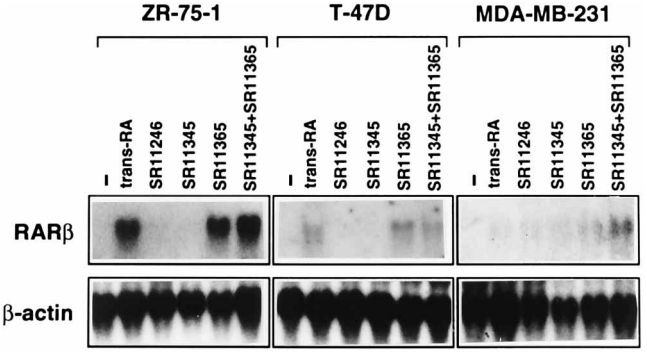

FIG. 3. Effects of RAR-selective SR11365 and RXR-selective SR11246 and SR11345 on RARβ gene expression in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and T-47D and *trans*-RA-resistant, estrogen-independent MDA-MB-231 cells. RNAs were prepared from cells treated with 10^{-6} M RXR-selective SR11246 or SR11345, RAR-selective SR11365, or a combination of SR11345 and SR11365 for 24 h and analyzed for RARβ expression by Northern blotting. For comparison, the expression of the β-actin is shown.

Competitive binding of RXR-RAR and RXR-nur77 heterodimers to the βRARE. Our observation that RARα inhibited the transactivation activities of RXR-selective SR11246 and SR11345 on the βRARE (Fig. 4B) suggests that RARα may compete with nur77 for heterodimerization with RXR and thus prevent nur77 from binding to the βRARE. We therefore carried out gel retardation assay using the βRARE as a probe (Fig. 5). When RARα protein was added, binding of RXRα-nur77 heterodimers to the βRARE decreased in a RARα dose-dependent manner. Excess amounts of RARα permitted binding of RXRα-RARα heterodimers. Similarly, increasing nur77 protein levels inhibited RXR-RAR heterodimer binding

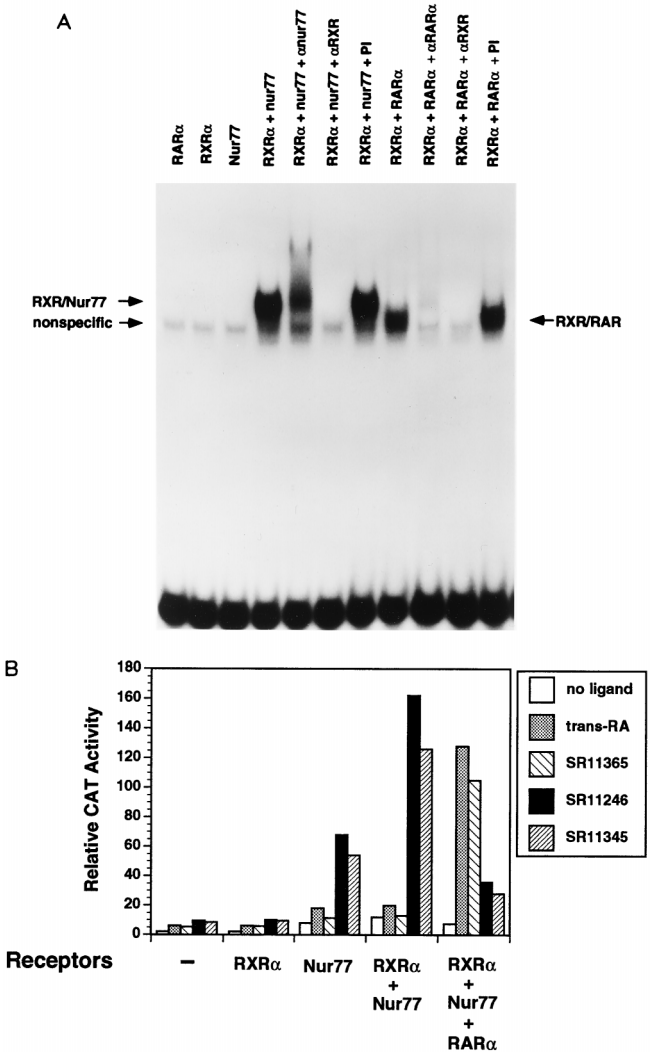

FIG. 4. Binding and transactivation of nur77-RXRα and RARα-RXRα on the βRARE. (A) Binding of the nur77-RXRα and RXRα-RARα heterodimers to the βRARE. Equal amounts of in vitro-synthesized nur77 and an N-terminally deleted RXRα (see Materials and Methods) alone or combined were incubated with the βRARE probe at room temperature for 10 min. Mixtures were analyzed by gel retardation. Anti-RXR (αRXR), anti-RARα (αRARα), or anti-nur77 (αnur77) was incubated with receptor proteins for 30 min at room temperature before performance of the assay. For a control, receptor proteins were also incubated with preimmune serum (PI). The βRARE probe sequence was GTAGGGTTCACCG**AAAGTTCA**GTC (the NBRE is in boldface). (B) nur-77-promoted transactivation of RARβ in CV-1 cells. A CAT reporter containing the RARβ promoter (19) was transiently transfected into CV-1 cells with the receptor expression vector RXRα (20 ng), nur77 (100 ng), or RARα (200 ng). After 24 h, cells were treated with the indicated retinoids (10^{-6} M) or *trans*-RA (10^{-7} M) for 24 h, and CAT activities were determined as described elsewhere (65).

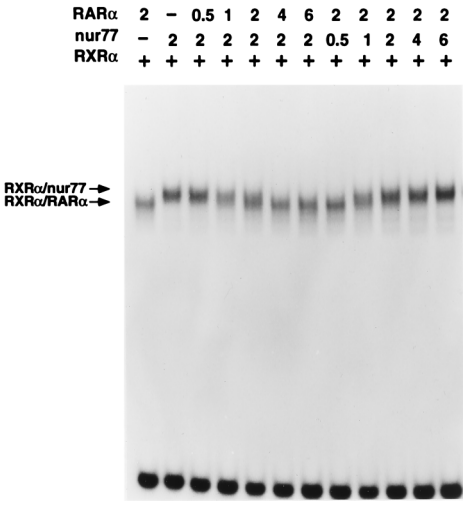

FIG. 5. Competition binding of RXR-RAR and RXR-nur77 heterodimers to the βRARE. To analyze the effect of RARα on RXR-nur77 heterodimer binding to the βRARE, in vitro-synthesized N-terminally deleted RXRα protein (1 μl) was incubated with in vitro-synthesized nur77 (2 μl) in the absence or presence of the indicated amounts (microliters) of in vitro-synthesized RARα protein and analyzed by gel retardation using the βRARE probe. To analyze the effect of nur77 on RXRα/RARα heterodimer binding, in vitro-synthesized RXRα protein (1 μl) was incubated with in vitro-synthesized RARα (2 μl) in the absence or presence of the indicated amounts (μl) of in vitro-synthesized nur77 and analyzed by gel retardation.

to the βRARE but enhanced RXR-nur77 heterodimer binding. Thus, RARα and nur77 compete for dimerization with RXR and binding to the βRARE. These data suggest that the relative levels of RARα and nur77 regulate binding of RXR-nur77 and RXR-RAR to the βRARE. We therefore determined whether different levels of RARα, RXRα, and nur77 were expressed in *trans*-RA-sensitive ZR-75-1 and in *trans*-RA-resistant MDA-MB-231 cells. Consistent with previous observations (34, 51, 54, 55, 60), RARα levels were much higher in ZR-75-1 cells than in MDA-MB-231 cells (Fig. 6). However, RXRα and nur77 were equally expressed in both cell lines independently of the presence of *trans*-RA. The high RARα levels in ZR-75-1 cells suggest that binding of RXRα-RARα heterodimers to the βRARE may preferentially occur to mediate the effects of RAR-selective ligands, while the low RARα levels in MDA-MB-231 cells suggest that RXR-nur77 heterodimer may be predominantly formed with the βRARE to mediate the inhibitory effects of RXR-selective ligands.

Stable expression of RARα in *trans*-RA-resistant MDA-MB-231 cells favors the RAR pathway over the RXR pathway. Low levels of RARα in MDA-MB-231 cells should enhance RXR-nur77 heterodimer formation to mediate the effects of RXR-selective retinoids. To determine whether overexpression of RARα would allow RXR-RAR heterodimer formation but inhibit that of RXR-nur77, we stably transfected RARα into

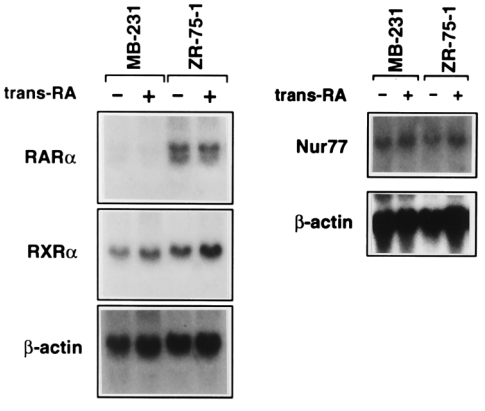

FIG. 6. Expression of RARα, RXRα, and nur77 in *trans*-RA-sensitive ZR-75-1 and *trans*-RA-resistant MDA-MB-231 cells. Total RNAs, prepared from cells treated with 10^{-7} M *trans*-RA for 24 h, were analyzed for the expression of RARα, RXRα, and nur77 by Northern blotting. For comparison, the expression of the β-actin is shown.

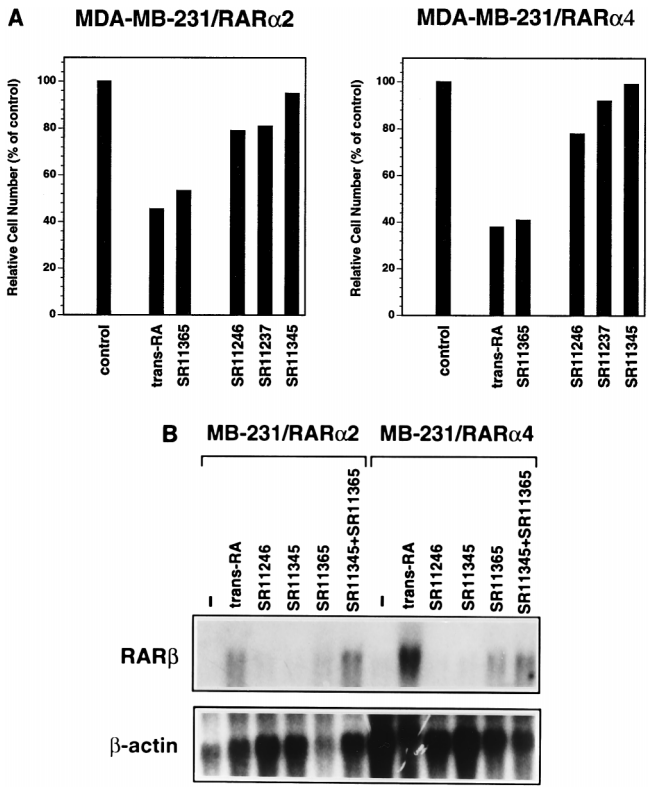

FIG. 7. Effect of stable expression of RARα on growth inhibition and RARβ induction by RXR-selective and RAR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells. (A) RARα modulates sensitivity of MDA-MB-231 cells to RAR-selective and RXR-selective retinoids. Stable clones, expressing introduced RARα, were seeded at 800 cells/well in 96-well plates and treated with the indicated retinoid (10^{-6} M) or *trans*-RA (10^{-7} M) for 10 days. The number of viable cells was determined by the MTT assay. (B) Stable expression of RARα regulates RARβ expression in response to RAR-selective and RXR-selective retinoids in MDA-MB-231 cells. Stable MDA-MB-231 clones expressing high levels of RARα (MB-231/RARα2 and MB-231/RARα4) were treated with the indicated retinoid (10^{-6} M) alone or in combination and analyzed for the expression of RARβ by Northern blotting. The expression of β-actin was used as the control.

this cell line. Two stable clones (MDA-MB-231–RARα2 and MDA-MB-231–RARα4) that expressed high levels of transfected RARα (data not shown) were analyzed for their responses to RAR and RXR class-selective retinoids (Fig. 7A). Compared to their effects in the parental MDA-MB-231 cells (Fig. 1), RAR-selective *trans*-RA at 10^{-7} M and SR11365 at 10^{-6} M were far more potent inhibitors of the stable clones, showing 46 to 62% inhibition, while RXR-selective SR11246, SR11237, and SR11345 were less effective inhibitors, with less than 21% inhibition. We also investigated the effect of RARα on RARβ expression in MDA-MB-231 cells by Northern blotting (Fig. 7B). In contrast to their effects on the parental cells (Fig. 3), RAR-selective *trans*-RA and SR11365 strongly induced RARβ expression in both clones, while RXR-selective retinoids SR11246 and SR11345 did not. These results demonstrate that low RARα expression in MDA-MB-231 cells is responsible for the increased ability of RXR-selective retinoids and the decreased ability of RAR-selective retinoids to induce RARβ expression and growth inhibition. The fact that the extent of growth inhibition and RARβ expression level by these receptor class-selective retinoids in the stable clones were similar to those in *trans*-RA-sensitive ZR-75-1 and T-47D cells (Fig. 1 and 3) suggests that the differential effects of retinoids on certain *trans*-RA-sensitive and -resistant breast cancer cell lines depend on different levels of RARα expression.

Binding of nuclear proteins from ZR-75-1 and MDA-MB-231 cells to the βRARE. To provide direct evidence that relative levels of RXR, RARα, and nur77 in *trans*-RA-sensitive ZR-75-1 and *trans*-RA-resistant MDA-MB-231 cells allowed different complex formation on the βRARE, we prepared nuclear proteins from ZR-75-1 and MDA-MB-231 cells and analyzed their binding to the βRARE (Fig. 8). Nuclear proteins

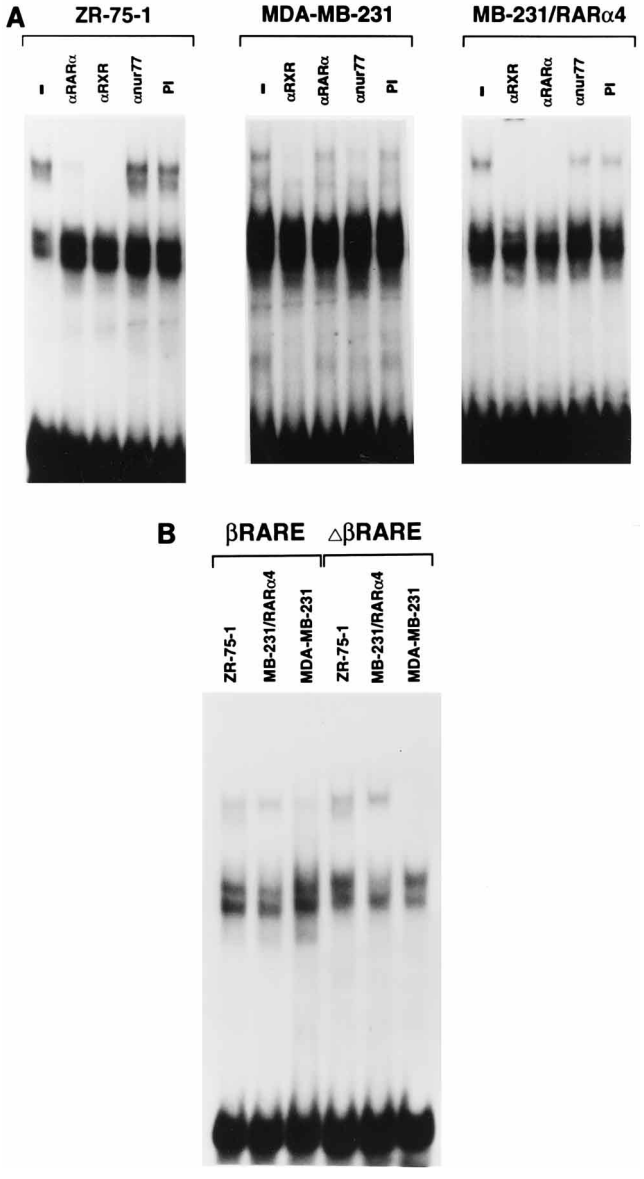

FIG. 8. βRARE binding activities of nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231–RARα4 cells. (A) Nuclear proteins from ZR-75-1 (2 μg), MDA-MB-231 (5 μg), and MDA-MB-231–RARα4 (2 μg) cells were analyzed by gel retardation assay using βRARE as a probe. When antibody (designated as in Fig. 4A) was used, it (1 μl) was incubated with nuclear proteins for 30 min at room temperature before performance of the assay. Preimmune serum (PI) was used for control. (B) Comparison of βRARE and ΔβRARE binding of nuclear proteins from ZR-75-1 (2 μg), MDA-MB-231 (2 μg), and MDA-MB-231/RARα4 (2 μg) cells by gel retardation assay. The ΔβRARE probe sequence was GTAGGGTTCACCG**TG**AGTTCAGTC (mutated nucleotides compared to βRARE are indicated in boldface).

from ZR-75-1 cells formed several strong complexes with the βRARE. When they were incubated with anti-RXR antibody, the slowly migrating complexes were inhibited. When anti-RARα antibody was used, some of the slowly migrating complexes were also abolished. However, anti-nur77 antibody did not show any detectable effect on the binding. These data demonstrate that RXR and RARα are mainly responsible for βRARE binding in ZR-75-1 cells. When nuclear proteins from MDA-MB-231 cells were analyzed, we observed weak βRARE binding complexes, which could be inhibited by anti-RXR and anti-nur77 antibodies but not by anti-RARα antibody. Thus, expression of RXR and nur77 in MDA-MB-231 cells (Fig. 6) could contribute to the βRARE binding activities. To determine whether overexpression of RARα in MDA-MB-231 cells could prevent RXR-nur77 binding as we observed by using in vitro-synthesized receptor proteins (Fig. 5), we analyzed the binding of nuclear proteins prepared from MDA-MB-231–RARα4 cells. As shown in Fig. 8A, the nuclear proteins formed a strong complex with the βRARE, which could be completely inhibited by either anti-RXR antibody or anti-RARα antibody but not by anti-nur77 antibody, indicating that overexpression of RARα in MDA-MB-231 cells inhibited RXR-nur77 heterodimer binding and permitted RXR-RAR heterodimer binding. To further determine the nature of the binding complexes from different cell lines, we used a mutated βRARE (ΔβRARE), in which two adenine nucleotides in the spacing region of the βRARE were mutated. The mutations do not affect binding of RXR-RAR heterodimers but abolish RXR-nur77 binding (66) and thereby allow distinction of RXR-RAR heterodimers from RXR-nur77 heterodimers. When nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231–RARα4 were analyzed on the ΔβRARE, we observed a strong binding of nuclear proteins from ZR-75-1 and MDA-MB-231–RARα4 cells, similar to that observed with the βRARE. In contrast, nuclear proteins from MDA-MB-231 cells did not show any detectable binding on the ΔβRARE, demonstrating that the binding complex that we observed on the βRARE might represent RXR-nur77 heterodimer binding.

DISCUSSION

Breast cancer cell growth inhibition and apoptosis induction by RXR-selective retinoids. Although conventional retinoids show promise in animal models as preventive agents against breast cancer, their anticancer effects appear to be limited to *trans*-RA-sensitive tumors, whereas the more aggressive, estrogen-independent tumors are usually refractory (14, 34, 54, 55, 60). In this study, we demonstrated that several RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1), provided that they are also capable of activating the RARs or are used in combination with RAR-selective retinoids. Efficient growth inhibition by RXR-selective retinoids appears to be cell type specific because it was observed in *trans*-RA-resistant MDA-MB-231 cells but not in *trans*-RA-sensitive ZR-75-1 cells (Fig. 1). In ZR-75-1 cells, RAR-selective SR11365 was much more effective than RXR-selective SR11246 in inhibiting the growth and inducing apoptosis (Fig. 1 and 2). Thus, different retinoid signaling pathways preferentially operate in *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cells to mediate retinoid-induced growth inhibition.

Although the RXR pathway is clearly involved in *trans*-RA-resistant MDA-MB-231 breast cancer cell growth inhibition, a clear growth inhibition by RXR-selective retinoids required a longer period of treatment (Fig. 1) than that by RAR-selective retinoids, which usually inhibit the growth of *trans*-RA-sensitive breast cancer cells over a period of 3 to 4 days of treatment (data not shown). This observation suggests that the effects of RXR-selective retinoids may involve a mechanism different from that utilized by RAR-selective retinoids. We showed that activation of RXR alone was insufficient for growth inhibition and that activation of RAR appeared to be required, as indicated by our observation that RXR-selective SR11237, SR11246, and SR11247, which at 1 μM have the ability to slightly activate the RARβ (Table 1), can significantly inhibit the growth of MDA-MB-231 cells (Fig. 1), whereas the far more RXR-selective SR11345, which activates RXRα comparably to the other RXR-selective retinoids (Table 1), did not significantly inhibit MDA-MB-231 cell growth (Fig. 1). Furthermore, RXR-selective SR11345 and RAR-selective SR11365, which alone were ineffective inhibitors, on combination strongly prevented MDA-MB-231 cell growth (Fig. 1). The biological activities of RXR-selective retinoids have been described in several studies (6, 17, 47). Activation of RXR was reported as essential for inducing apoptosis in HL-60 leukemia cells (47). RXR-selective retinoid LGD1069 effectively inhibited the tumor development in the *N*-nitroso-*N*-methylurea-induced rat mammary tumor model (17). Because of their increased efficacy against malignant, *trans*-RA-resistant, estrogen-independent breast cancer growth, RXR-selective retinoids may be useful for chemoprevention and chemotherapy of breast cancer.

How RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 and other cancer cells remains to be fully elucidated. Induction of growth inhibition and apoptosis of breast cancer cells by retinoids may involve different retinoid receptors and different mechanisms, depending on types of retinoids and cell lines (11, 12, 33, 34, 53–55, 60). The effects of *trans*-RA may be mediated by RARα and RARβ (33, 34, 53–55, 60), whereas activation of RARγ may be required for other retinoids, such as 4-HPR (11, 12). Our data presented here suggest that induction of RARβ may be involved. This is consistent with previous observations that RARβ could mediate the growth-inhibitory effect of *trans*-RA in breast cancer cells (33, 34, 53). RARβ was induced by *trans*-RA only in *trans*-RA-sensitive ZR-75-1 and T-47D, not in *trans*-RA-resistant MDA-MB-231, breast cancer cells (34). In addition, introduction of RARβ into RARβ-negative MDA-MB-231 breast cancer cell lines restored *trans*-RA-induced growth inhibition (33, 34, 53), while inhibition of RARβ activity in the RARβ-positive ZR-75-1 cells with an antisense construct abolished growth inhibition by *trans*-RA (34). Furthermore, enhancement of RARβ levels has been found to correlate with senescence in normal mammary epithelial cells (58). In *trans*-RA-sensitive ZR-75-1 and T-47D cells, RARβ expression was strongly induced by RAR-selective *trans*-RA and SR11365, which also inhibited growth and induced apoptosis, whereas RARβ expression was not induced by RXR-selective SR11246 and SR11345, which only poorly inhibited growth and only weakly induced apoptosis (Fig. 3). The fact that RXR-selective retinoids could induce RARβ expression in *trans*-RA-resistant MDA-MB-231 cells (Fig. 3) suggests that induction of RARβ may contribute to their effects on MDA-MB-231 cells. However, because *trans*-RA and SR11365 induced RARβ to levels similar to those induced by RXR-selective retinoids SR11246 and SR11345 in MDA-MB-231 cells (Fig. 3) but were poor growth inhibitors (Fig. 1), mechanisms other than RARβ induction may also be involved.

SR11345 synergized with RAR-selective retinoids to inhibit MDA-MB-231 cell growth (Fig. 1). Such synergism of RAR- and RXR-selective retinoids has recently been observed in the

activation of several RA-responsive genes, including RARβ, during embryonal carcinoma cell differentiation (52) and in NB4 acute promyelocytic leukemia cells (4). The synergism that we observed here may in part arise from induction and activation of RARβ. Growth inhibition of MDA-MB-231 cells may require both induction and activation of RARβ because RXR-specific SR11345 is a much less effective inhibitor than other RXR-selective retinoids (Fig. 1), which also slightly activate RARβ (Table 1). Enhanced induction of RARβ in MDA-MB-231 cells by the combination of RXR-specific SR11345 and RAR-selective SR11365 (Fig. 3) may also contribute to their synergistic growth inhibition.

Regulation of RARβ expression. The βRARE in the RARβ promoter is responsible for regulating RARβ expression by retinoids (10, 21, 57). Our observation in gel shift assays that efficient binding to the βRARE occurred by heterodimerization of RXRα with nur77 or RARα but not by either receptor alone (Fig. 4A) confirms that the βRARE binds both RXR-RAR and RXR-nur77 heterodimers (15, 49). Activation of the βRARE by *trans*-RA is caused by binding and activation of RAR-RXR heterodimers, in which RXR functions as a silent partner (15, 30, 38), while activation of the βRARE by RXR-selective retinoids occurs on binding of RXR-nur77 heterodimers (15, 49). Cotransfection of RXRα and nur77 strongly activated RARβ promoter activity in response to RXR-selective SR11246 or SR11345 but not to RAR-selective *trans*-RA or SR11365 (Fig. 4B). Thus, the βRARE can be activated by either a RAR-selective or RXR-selective retinoid signaling pathway through binding of RXR-RAR or RXR-nur77, respectively. This is reminiscent of a previous observation that the βRARE bound strongly to an RXR-containing complex other than RXR-RAR in S91 melanoma cell extracts (56). Because nur77 expression is induced by several growth factors having different signal transduction pathways (20, 39), the binding and activation of the βRARE by nur77 and retinoid receptors will mediate the convergence of retinoid and growth factor signaling pathways.

Activation of the βRARE by the RAR pathway or RXR pathway depends on the breast cancer cell type. In *trans*-RA-sensitive cell lines such as ZR-75-1 and T-47D, the expression of RARβ was highly induced by RAR-selective retinoids but not by RXR-selective retinoids, whereas in *trans*-RA-resistant MDA-MB-231 cells, RARβ expression was induced by RXR-selective retinoids (Fig. 3). Because both RXRα and nur77 are well expressed in MDA-MB-231 cells (Fig. 6), induction of RARβ by RXR-selective retinoids is likely mediated by activation of RXR-nur77 heterodimers on the βRARE. This is further supported by our observation that binding of nuclear proteins prepared from MDA-MB-231 cells to the βRARE may represent RXR-nur77 heterodimers (Fig. 8). Such cell-type-specific activation of the βRARE has been observed previously. In CV-1 cells, RXR-selective retinoids did not appreciably transactivate the DR-5 βRARE even in the presence of transfected RAR and RXR (15, 30). However, in P19 or F9 embryonal carcinoma cells, RXR ligands contributed to transcriptional activation of genes containing DR-5 elements (4, 40, 52). The cell-type-specific activation of the βRARE is likely due to the relative levels of nuclear receptors that bind the βRARE and modulate its activity.

Although RXR-selective retinoids could induce RARβ expression in MDA-MB-231 cells, we observed a stronger induction of RARβ when cells were treated with a combination of RAR-selective and RXR-selective retinoids (Fig. 3). The strong induction of RARβ by the combination may be due to the additive effect of RXR-RAR and RXR-nur77 heterodimers, since RAR-selective retinoids by themselves could also slightly induce RARβ probably due to low levels of RARα expressed in these cells. Recently, it was reported that binding of RAR-selective retinoids to RXR-RAR heterodimers allowed binding and activation of RXR-RAR heterodimers by RXR-selective retinoids (4, 40). Thus, it is also possible that the strong induction of RARβ that we observed by the combination of RAR-selective and RXR-selective retinoids is due to activation of RXR-RAR heterodimers prebound with RAR-selective retinoid.

RARα regulates both RAR and RXR pathways. *trans*-RA-sensitive and -resistant breast cancer cell lines display different responses to retinoid receptor class-selective ligands. RAR-selective retinoids are potent RARβ inducers and growth inhibitors in *trans*-RA-sensitive ZR-75-1 cells, while RXR-selective retinoids effectively induce RARβ and inhibit the growth of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1 and 3). The observation that the βRARE can be activated by either RXR-RAR or RXR-nur77 suggests that the pathway that mediates growth inhibition and RARβ induction may largely depend on the relative levels of RARα, RXRα, and nur77. In lung cancer cell lines, nur77 expression is associated with *trans*-RA resistance (66) and could be critical in regulating RAR and RXR activities. However, in breast cancer cell lines, ZR-75-1 and MDA-MB-231 cell lines express similar levels of RXRα and nur77, while RARα varies, being highly expressed in the former and underexpressed in the latter (Fig. 6), as has been previously observed (34, 51, 55, 60). Thus, RARα levels appear to be most important for determining whether the RAR or RXR pathway will regulate growth inhibition by retinoids. High RARα levels in *trans*-RA-sensitive ZR-75-1 cells may permit formation of RXR-RAR heterodimers that bind to the βRARE (Fig. 5 and 8) to mediate the effects of RAR-selective retinoids in inducing RARβ expression and growth inhibition (34) but prevent RXRα from forming RXR-nur77 heterodimers (Fig. 5 and 8) so that RXR-selective retinoids are unable to inhibit growth or induce apoptosis despite the abundant expression of nur77. In contrast, low RARα levels in *trans*-RA-insensitive MDA-MB-231 cells (Fig. 6) allow formation of RXR-nur77 heterodimers (Fig. 8) that bind to the βRARE to mediate RARβ expression and may be responsible for growth inhibition in the presence of RXR-selective retinoids. The importance of RARα levels in determining the regulatory pathway is supported by our gel retardation (Fig. 5 and 8) and transfection assay results (Fig. 4B). Gel retardation indicates that binding of RXR-RAR or RXR-nur77 heterodimers to the βRARE largely depends on RARα protein levels (Fig. 5 and 8). Overexpression of RARα in MDA-MB-231 cells allowed binding of RXR-RARα heterodimers and prevented binding of RXR-nur77 heterodimers to the βRARE (Fig. 5 and 8). In transient transfection assays, cotransfection of RARα inhibited RXR-selective retinoid-induced RXR-nur77 heterodimer activity on the RARβ promoter (Fig. 4B). Furthermore, stable expression of RARα in MDA-MB-231 cells strongly enhanced growth inhibition (Fig. 7A) and RARβ induction (Fig. 7B) by RAR-selective retinoids and decreased the inhibitory effects of RXR-selective retinoids. Thus, high RARα levels favor formation of RAR-RXR heterodimers and the RAR signaling pathway in breast cancer cells, while low RARα levels favor the formation of nur77-RXR heterodimers and the RXR signaling pathway. This retinoid signaling switch may play an important role in regulating breast cancer cell growth in response to different growth factor and retinoid stimuli.

In summary, we have demonstrated that RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 breast cancer cells, which appears to

be mediated through RXR-nur77 heterodimers that bind and activate the βRARE in the presence of RXR-selective retinoids, resulting in induction of RARβ, which may then be activated by RAR-selective retinoids to initiate secondary biological responses. RXR-nur77 heterodimer formation in *trans*-RA-resistant MDA-MB-231 cells is favored by very low RARα levels, whereas high expression of RARα in *trans*-RA-sensitive ZR-75-1 cells favors formation of RXR-RAR heterodimers that bind and activate the βRARE in response to RAR-selective ligands. Thus, the convergence and switch of RAR-dependent and RXR-dependent signaling on the βRARE is very likely regulated by relative RARα levels. Our findings that an RXR signaling pathway can mediate growth inhibition and apoptosis induction and the additive to synergistic effects of a RAR-selective and RXR-selective retinoid combination on *trans*-RA-resistant MDA-MB-231 cell growth may provide a therapeutic opportunity to inhibit the growth of more invasive, *trans*-RA-resistant breast cancer by using lower retinoid doses to reduce toxicity.

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