Overexpressed Activated Retinoid X Receptors Can Mediate Growth Inhibitory Effects of Retinoids in Human Carcinoma Cells*

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Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) mediate the effects of retinoids on gene expression by binding to response elements in retinoic-sensitive genes. RAR- but not RXR-selective retinoids were found in many previous studies to suppress the growth of various cells, implicating RXR-RAR in these effects. Using a co-expression vector for identifying cells that expressed retinoid receptors transiently and 5'-bromo-2'-deoxyuridine incorporation for labeling DNA-synthesizing cells, we found that RXR-selective retinoids inhibited DNA synthesis in squamous carcinoma 1438 cells transfected with RXRα but not with RARs. Ligand-induced transcription of the reporter luciferase gene via the activation of RXR-RXR but not RXR-RAR correlated with growth suppression. Studies with RXRα deletion mutants indicated that the DNA binding and the ligand binding domains are essential for mediating growth inhibition. A point mutation in the ligand binding domain (L430F) that decreased RXRα homodimerization compromised its growth inhibitory function. Further, RXRα mutant (F313A), which functions as a constitutively active receptor, inhibited DNA synthesis in the absence of ligand. These results demonstrate that RXR homodimer activation leads to growth inhibition and suggest that transfection of RXRα and treatment with RXR-selective retinoids or the transfection of constitutively activated RXRα mutant alone may have a therapeutic potential.

Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are nuclear retinoid receptors, which belong to the superfamily of steroid hormone receptors (reviewed in Refs. 1–3). Like other members of this superfamily, retinoid receptors have a conserved structure of domains A to F from the N terminus to the C terminus of the molecule, respectively. These domains have distinct functions and can act independently (1–3). The N terminus of the receptors (domains A and B) contains an autonomously functioning region called activation function 1 (AF-1), which is involved in ligand-independent transcriptional transactivation and is not well conserved among receptors. Domain C, a DNA binding domain composed of two class II zinc-binding motifs, is highly conserved. Domain D (hinge) is involved in ligand-induced functional changes and in the binding of receptors to co-repressors. Domains E/F, which are moderately conserved among the receptors, are thought to be involved in ligand-binding, ligand-dependent transactivation function 1 (AF-1) and dimerization (3). RARs bind both natural retinoids all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid (9-cis-RA), whereas the RXRs bind only 9-cis-RA (1–3). Some synthetic retinoids can bind selectively to either RARs or RXRs or their α, β, or γ subtypes (4–6).

Retinoid receptors modulate the expression of their target genes by interacting as either homodimers or heterodimers with specific DNA response elements (1–3). The affinity of RARs for their target sequence is increased on heterodimerization with RXRs. RXRs dimerize with and enhance the transcriptional activity of not only RARs but also thyroid hormone receptor, vitamin D receptor, peroxisome proliferator-activated receptor, and several orphan receptors (3, 7). RXR can also form homotetramers and homodimers, and, in the presence of 9-cis-RA, RXR-RXR dimers can activate gene transcription (1–3, 7–11).

Retinoic acid and retinoid X response elements (RARE and RXRE, respectively) are commonly composed of two half sites, each of which provides a binding site for one of the receptor molecules in a dimer. Each of the half sites is a conserved hexanucleotide DNA sequence, 5'-PuG(G/T)TCA-3', and the two form direct repeats (DR) separated by one to five nucleotides (1–3). Both the relative orientation and spacing of the half sites is important for receptor recognition and for the subsequent activation or repression of the expression of target genes (12–15). For example, in the presence of ligand, RXR-RAR heterodimers bind to and activate transcription on response elements consisting of two direct repeats separated by five nucleotides (DR-5), whereas RXR-RXR heterodimers bind direct repeats spaced by one nucleotide (DR-1) to elicit constitutive repression of gene transcription. In the presence of 9-cis-RA the RXR-RXR homodimer can activate gene transcription from the DR-1 response element (8). The function of retinoid receptors is also regulated by co-activators and co-repressors that distinguish among different conformations of dimer-DNA complexes induced by ligand binding and dimerization and consequently modulate positively or negatively the expression of target genes by retinoid receptors (3, 16–18).

Retinoids were found to be effective inhibitors of cancer development in animal models of carcinogenesis and to suppress premalignant lesions and the development of second pri-

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§ The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; RXRE, retinoid X response element; ATRA, all-trans-retinoic acid; 9-cis-RA, 9-cis-retinoic acid; DR, direct repeat; HNSCC, head and neck squamous cell carcinoma; BrdU, 5'-bromo-2'-deoxyuridine; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8,-tetratomethyl-2-naphthalenyl)-1-propenyl]benzoic acid.
RXR-Mediated Inhibition of Tumor Cell Growth

The expression of certain RARs (e.g., RARβ) is decreased in various tumor cell lines, and retinoid responsiveness could be restored by stably transfecting receptor expression vectors (28–30). However, the isolation of stable transfectants expressing genes whose products exert growth-inhibitory effects may be difficult and, even if successful, may yield cells that were already partially or completely resistant to the inhibitory effect of this gene product. This problem can perhaps be solved by an inducible promoter or by transient expression of the gene. Whereas the transient transfection approach can provide results much faster than the stable transfection approach, its use is counterindicated when the efficiency of transfection is low. This limitation can be overcome by a method that allows the identification of the cells that express the transfected gene. A single cell proliferation assay has been developed to study the growth-inhibitory function of a candidate gene in a transient expression assay (31). This assay is based on the pMark vector engineered to co-express a nuclear retinoid receptor and a cell surface antigen (e.g., CD7), which permits identification of transfected cells bearing the antigen using a fluorescently labeled anti-CD7 antibody (31). Growth inhibition is then determined by labeling DNA-synthesizing nuclei with 5-bromo-2'-deoxyuridine (BrdU) followed by staining with anti-BrdU antibodies labeled with a different fluorochrome (31). We used the pMark vector to express four nuclear retinoid receptors, RARα1, RARβ2, RARγ1, and RXRα, in head and neck squamous carcinoma (HNSCC) 1483 cells and then determined the effects of the receptor expression on DNA synthesis in the cells grown in the absence or presence of different receptor-selective retinoids. After we found RXRα to be the most effective among these receptors in suppressing DNA synthesis, we focused on characterizing the effect of RXRα expression and the role of the different receptor domains on the growth and retinoid responsiveness of HNSCC 1483 cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Retinoid Treatment—HNSCC 1483 cell line (32) was maintained in Dulbecco’s modified Eagle’s essential medium containing 10% fetal bovine serum. Cells were incubated at 37 °C in humidified 5% CO₂, 95% air. Cells were detached by repeated pipetting after a brief incubation with 2 mM EDTA and 0.25% trypsin in a calcium-free and magnesium-free air. Cells were detached by repeated pipetting after a brief incubation with 0.5 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin) on ice for 60 min, followed by ultracentrifugation (100,000 × g, 20 min at 4 °C). The supernatant was dialyzed against buffer C (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10% glycerol, 26916 g/μM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin) for 16 h on ice. The nuclei were collected by centrifugation (6000 × g, 5 min at 4 °C), solubilized in 3 volumes of buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 600 mM KCl, 1 mM dithiothreitol, 10 mM monothioglycerol, 1 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin) on ice for 30 min. After centrifugation (100,000 × g, 20 min at 4 °C), the supernatant was collected by centrifugation (2000 × g, 10 min at 4 °C), and the modified RXRα construct was recovered by adjusting the protein concentration to 1 mg/ml in buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 600 mM KCl, 1 mM dithiothreitol, 10 mM monothioglycerol, 1 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin) for 4 h. The synthetic oligonucleotides indicated below, which represent the DR-5 RARE and flanking sequences present in the RARβ2 gene (35) (5'-TCGAGGTAGTTGAGCAAAAATCTG-3' and 3'-AGC-TCCATCCCAAAGTGGGTTTCAAGTGAGG-5'), were labeled with [γ-32P]ATP (4000 Ci/mM; ICN, Irvine, CA) using T4 polynucleotide kinase. Nuclear extracts were preincubated with 2 μg of poly(dI-dC)poly(dI-dC) for 15 min at 4 °C and then incubated with labeled DNA (approximately 10,000 cpm) for 15 min at 4 °C in the presence of 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 1% dithiothreitol, 5 mM MgCl₂, and 20% glycerol. For supershift analysis, receptor-specific monoclonal antibodies 8B8R (RARa), Ab8B (RARb), and Ab4G and Ab4G (RXRs), 4X RXRα, 4RX RXRβ, and polyclonal antibody RP (obtained from Dr. Pierre Chambon (IGBM, Illkirch, Strasbourg, France)) (33, 34) were added (0.5 μl) to the 20-μl reaction mixture before electrophoresis. The reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel containing 25 mM Tris-HCl buffer, pH 8.5, 192 mM glycin, 1 mM EDTA. All of the above antibodies were capable of supershifting the respective receptors using nuclear extracts from at least one cell type in our laboratory (data not shown).

- Plasmids—Construction of Plasmids—Human cDNAs for RARα1, RARβ2, RARγ1, and RXRα were obtained from Dr. Magnus Pfahl (Sidney Kimmel Cancer Center, San Diego, CA). The cDNAs were released from pBluescript vectors by digesting with BamHI and HindIII or NotI and HindIII restriction enzymes and blunted with the Klenow fragment of DNA polymerase I and then inserted into the Smal site of the plasmid pMarkCD7Δ5 (Ref. 31, produced by the Genetics Institute, Cambridge, MA), which was obtained from Dr. Jonathan Kurie (Department of Head and Neck/Thoracic Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX). The orientation of the inserted cDNAs was identified by restriction enzyme digestion. The resulting expression vectors were named pMarkRARα, pMarkRARβ, pMarkRARγ, and pMarkHXRα, respectively. RXRα deletion mutants (shown schematically in Fig. 4A) were prepared in the plasmid pBSRXRα, and the modified RXRα cDNAs were released and inserted into pMarkCD7Δ5. For example, the mutant RXRαL418F was prepared by deleting the part of the 5' end of wild-type RXRα cDNA with HindIII and Smal, and then the vector was blunt-ended by T4 DNA polymerase and self-ligated. Mutant RXRαL4Δ was prepared by deleting the fragment between the first and the third NotI sites (nucleotides 29–197) in wild-type RXRα cDNA. Mutant RXRαL4Δ was prepared by deleting the 3' end of the RXRαL4Δ from the first StuI site (nucleotide 402). RXRα point mutants are shown schematically in Fig. 5. pMarkRXRα F318A, a homologue of mouse RXRα F318A (36), was constructed using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The primers used for generation of this mutation were as follows: sense primer, 5’-GTCGTC TAGAT CCCT CGCCT CCAC CCAC CCTG CTCG C-3’; antisense, 5’-GGCAT GAGCC GACCG GAGCG GATG AGCAG C-3’. pMarkRXRα L1418F, pMarkRXRα L430F, and pMarkRXRα K314Q were prepared as follows: pBSRXRα L1418F, pBSRXRα L430F, and pBSRXRα K314Q (37) (provided by Dr. Xiao-kun Zhang, Burnham Cancer Institute, La Jolla, CA) were digested with HindIII and NotI. The released fragments were blunted by...
Klenow and then ligated into the Smal site of pMarkCD7Δ5. The success of obtaining the desired deletions and point mutations was assessed by sequence analysis.

Single Cell Proliferation Assay—The assay was performed as described by Frangioni et al. (31) with modifications. HNSCC 1483 cells were seeded at a concentration of 10⁵ cells per well in 6-well plates. After 18–24 h, cells were transfected with various pMark vectors using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s instructions. Each well received 1 µg of plasmid DNA and 6 µl of LipofectAMINE. After 6 h, the transfection solution was removed by aspiration, and the cells were refed with medium containing 10% dextrose serum and the indicated concentration of retinoids or MeSO control. After 36 h, the cells were incubated for 8.5 h with a labeling reagent containing 10 µM BrdU and 1 µM 5'-fluoro-2'-deoxyuridine (Amersham Pharmacia Biotech) to incorporate BrdU into DNA in cells engaged in DNA synthesis. Cells were then washed three times with PBS and fixed by the gentle addition of absolute methanol (prechilled to −20 °C) for 10 min. Cells were then rehydrated with PBS and washed once in water. Chromosomal DNA was depurinated by treatment with 2 M HCl for 15 min at room temperature. The acid was neutralized by one wash with 0.1 M Na₂B₄O₇, pH 8.5, followed by a 2-min incubation in the same solution. Cells were then washed twice with 0.1% Nonidet P-40 in PBS and incubated for 1–2 h at room temperature with anti-BrdU monoclonal antibody (IgG1, Becton Dickinson) and anti-CD7 monoclonal antibody (IgG2b clone no. 3A1E-12H7, Sera-Lab, Sussex, United Kingdom), both diluted 1:6 in 0.3% bovine serum albumin and 0.1% Nonidet P-40/PBS. The cells were washed five times with 0.1% Nonidet P-40/PBS and incubated for 45 min at room temperature with Texas Red-conjugated goat anti-mouse IgG2b and fluorescein isothiocyanate-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) diluted 1:200 in 0.1% Nonidet P-40/PBS. Cells were then washed four times with 0.1% Nonidet P-40/PBS and twice with PBS and observed using an immunofluorescence microscope with filters for the red fluorescence of Texas Red in the cytoplasm and on the cell surface and the green fluorescence of fluorescein isothiocyanate in the cell nuclei. Cells that have taken up the plasmid and synthesized DNA stained red and green (RG). Cells that have taken up the plasmid but failed to synthesize DNA stained only red (R). The BrdU labeling index (percentage of cells synthesizing DNA among the cells that have taken up the plasmid) was determined from the formula (RG/ (R + RG))/100. Usually, a total population of over 500 cells was analyzed in several arbitrarily chosen microscopic fields.

Transient Transfection and Luciferase Assays—Cells were seeded at a concentration of 1.5 × 10⁵ cells per well in 6-well plates. After overnight culture, cells in each well were transfected with 2 µg of DNA including 1.5 µg of reporter plasmid, 0.1 µg of pCH110, and 0.4 µg of pMark or pMark-derived expression vectors using 6 µl of LipofectAMINE (Life Technologies, Inc.) using the manufacturer’s procedure. The reporter plasmids included the (RARE)₃-tk-LUC, which contains three direct repeats of DR-5 RARE from the P2 promoter region of the human RAR β gene (from −59 to −53 base pairs) (35) connected to the minimal herpes simplex virus thymidine kinase promoter and a luciferase cDNA and the RXRE-tk-LUC, which contains five tandem repeats of a 35-base pair sequence (DR-1) from the promoter of the human RAR α gene (from −59 to −53 base pairs) (35) connected to the minimal herpes simplex virus thymidine kinase promoter and a luciferase cDNA. The reporter plasmid pMarkCD7Δ5 was used as the internal control for transfection efficiency.

RESULTS

Endogenous Retinoid Receptors in HNSCC 1483 Cells Can Mediate Transcriptional Activation of DR-5 RARE but Not DR-1 RXRE—Previous studies demonstrated that HNSCC 1483 cells express mRNAs for each of the RAR and RXR subtypes (α, β, and γ) (39). The very low level of RARγ increased on

FIG. 1. Functional analysis of endogenous nuclear retinoid receptors in 1483 cells. A. Identification of endogenous nuclear retinoid receptors in 1483 cells that form complexes with the DR-5 RARE. Nuclear extracts were prepared from cells treated for 6 days with either MeSO (lanes 1–7) or 1 µM 9-cis-RA (lanes 8–14). Gel shift and supershift analyses were performed as described under “Experimental Procedures.” The name and the receptor specificity of the antibodies used are indicated above each lane. Shifted band and supershifted bands I, II, and III corresponding to RARα, RXR (antibody does not distinguish different isotypes), and RARβ (right arrows on left). Although RARα was not shifted in the 1483 nuclear extract, the antibody AB96 was capable of supershifting RARα in extracts of another HNSCC (data not shown). B. 9-cis-RA activates DR-5 RARE but not DR-1 RXRE. Cells were transfected with luciferase reporter constructs containing either the DR-5 or DR-1 response element and with a β-galactosidase expression vector as described under “Experimental Procedures.” The cells were then treated with either 0.01% MeSO (DSMO) or 1 µM 9-cis-RA in MeSO for 20 h. The cells were harvested and analyzed for luciferase and β-galactosidase activities. Data are presented as the activity of luciferase normalized to that of β-galactosidase, which served as a control for transfection efficiency.

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decreased by 70% in cells transfected with RXR but not DR-1 in pMarkRXR bind to RXRs (40), increased reporter gene transcription from to be associated. The exogenous RXR transfected cells. On treatment with 1 m concentration of 9-cis-RA 9–12-fold in RXR produced DR-1 activity only in RXR heterodimer pathway functioned in the 1483 cells. Indeed, gel shift and supershift analyses performed with the RARβ DR-5 RARE (Fig. 1A) revealed that after 9-cis-RA treatment, RARβ (lane 11), RARγ (lanes 12 and 13), and RXR (lane 14) proteins complexed with DR-5. Complexes formed with DR-5 by proteins in nuclear extracts from untreated cells also contained RARγ (lanes 5 and 6) and RXRα (lane 7), but the level of RARβ was apparently too low to be observed in the supershift analysis (Fig. 1A, lane 4). A functional assay for endogenous receptors was performed by transient transfection of reporter plasmids containing either DR-5 RARE or DR-1 RXRE. 9-cis-RA at 1 μM increased the transcription of luciferase via DR-5 RARE by about 10-fold but failed to enhance the transcriptional activity via DR-1 RXRE (Fig. 1B). These findings suggest that the endogenous RXR-RAR heterodimer is functional and can be activated by 9-cis-RA in the 1483 cells, whereas the endogenous RXR-RXR homodimer is not.

The Expression of an Exogenous RXRα Increases the Activation of RXR Homodimer and Suppresses DNA Synthesis in 1483 Cells after Treatment with 9-cis-RA—The formation of RXR-RXR homodimers requires high RXR levels (10, 11). Thus, one reason for the lack of activation of the RXR homodimer pathway in the 1483 cells could be a low level of endogenous RXR. Therefore, we transiently transfected the 1483 cells with pMarkCD7.5 (pMark in short) vectors harboring RXRα as well as RARα, RARβ, and RARγ for comparison. We found that only cells transfected with RXRα exhibited increased growth inhibition after treatment with 1 μM 9-cis-RA, whereas the other transfected receptors failed to increase suppression of DNA synthesis by the same retinoid relative to cells transfected with pMark vector only (Fig. 2A). These results suggested that an increase in RXR homodimers but not RXR-RAR heterodimers contributes to growth inhibition by 9-cis-RA.

Further support for this conclusion has come from analysis of transcription activation experiments. Transfection of pMarkRXRα followed by treatment with 1 μM 9-cis-RA induced luciferase transcription 10-fold via DR-1 RXRE as compared with almost no induction in pMark vector only transfected cells (Fig. 2B). Lower concentrations of 9-cis-RA (e.g. 0.1 or 10 nM) failed to activate DR-1 in RXRα-transfected cells. A similar dose dependence was found for the effects of 9-cis-RA on the BrdU labeling index in transiently transfected cells. On treatment with 1 μM 9-cis-RA, DNA synthesis decreased by 70% in cells transfected with RXRα, whereas treatment with 0.1 or 10 nM 9-cis-RA had no effect (Fig. 2C). Thus, the transactivation of RXRE and inhibition of DNA synthesis seemed to be associated. The exogenous RXRα expression also increased luciferase transcription through DR-5 RARE, presumably by stimulating the RXR-RAR pathway; however, this increase was by less than 50% (Fig. 2B).

The Ability of RXR-selective Retinoids to Inhibit the Growth of 1483 Cells Is Related to Their Ability to Activate Transcription of DR-1 RXRE in RXRα-transfected 1483 Cells—Because 9-cis-RA can bind both RARs and RXRs and activate transcription of both the RXR-RAR and the RXR-RXR pathways, we used RXR-selective TTNPB and several RXR-selective retinoids to determine whether they exert distinct effects on transcription of reporter genes and growth in 1483 cells transfected with pMarkRXRα or pMark vector alone. TTNPB, which binds to RARs with 10-fold higher affinity than ATRA but does not bind to RXRs (40), increased reporter gene transcription from DR-5 but not DR-1 in pMarkRXRα-transfected cells (compare panels A and B in Fig. 3). 9-cis-RA increased DR-5 activity in both vector only- and RXRα-transfected cells but strongly induced DR-1 activity only in RXRα-transfected cells. RXR-selective SR11217, SR11203, SR11234, and SR11246 activated DR-1 9–12-fold in RXRα-transfected cells (Fig. 3B) but only increased DR-5 activity less than 2-fold (Fig. 3A). These results indicate that the RXR-selective retinoids activated the RXR-RXR pathway in RXRα-transfected cells. RXR-selective TT-
NPB failed to suppress DNA synthesis in RXRα-transfected cells, whereas the RXR-selective retinoids inhibited DNA synthesis by 50–80%, and their potency seemed to be related to their efficacy in DR-1 activation (Fig. 3C). These findings suggest that activating RXR-RAR pathway alone is not sufficient to attenuate cell growth, whereas activation of DR-1 (presumably by RXR homodimers) in RXRα-transfected cells can inhibit growth.

**RXRα Deletion Mutants Differ in Their Ability to Mediate Inhibition of DNA Synthesis after Treatment with 9-cis-RA**—To investigate the importance of various regions of RXRα in inhibition of DNA synthesis by 9-cis-RA, we prepared deletion mutants of RXRα in the pMark vector (Fig. 4A). Their activities were compared with those of wild-type RXRα in transfected 9-cis-RA-treated cells. A mutant with deletion of part of domain A (RXRαΔA) was as effective as wild-type receptor in activating transcription from DR-5 but 35% less effective in activating transcription from DR-1 (Fig. 4, B1 and B2). The RXRαΔA mutant was less potent than the wild-type receptor in mediating suppression of DNA synthesis (54 and 72% inhibition for mutant and wild type, respectively) (Fig. 4B3). RXRα mutants with a deletion of a large part of the A/B domain and the DNA binding domain (RXRαΔD) or a 61-amino acid deletion at the C terminus (RXRαΔF) had decreased ability to activate DR-5 (the reason why the activity is not decreased to zero may be due to the presence of active endogenous RXRα (39)) and no ability to activate DR-1 (Fig. 4, B1 and B2). These mutants also lost the ability to suppress DNA synthesis in the presence of 9-cis-RA (Fig. 4B3). These findings, therefore, demonstrate a relationship between ability of RXRα to transactivate DR-1 and to mediate growth inhibition.

**RXRα Mutants with a Reduced Ligand-induced Homodimerization Activity Decreased Growth Inhibition by 9-cis-RA**—The
RXR-mediated Inhibition of Tumor Cell Growth

Several studies have shown that RAR-selective retinoids effectively suppress tumor cell growth or induce cell differentiation, whereas RXR analogs are less effective (6, 23, 24). These findings suggested that the RXR-RAR pathway mediates the effects of retinoids in these cell systems, whereas RXR-RXR homodimers do not play a role. There are, however, reports that RXR-selective retinoids can mediate induction of certain genes (41–43). Some of these genes and other genes regulated by RXR signaling may be involved in growth control.

We found that retinoid signaling in the untransfected HNSCC 1483 cells is mediated primarily by the RXR-RAR heterodimer pathway as indicated by the activation by 9-cis-RA of DR-5 RARE but not DR-1 RXRE (Fig. 1B). Indeed, a DR-5 regulated gene, RARβ (35), was induced by 9-cis-RA in the 1483 cells as indicated by the appearance of a supershifted band using anti-RARβ antibodies after 9-cis-RA treatment of 1483 cells (Fig. 1B). This finding was also supported by our recent observation that both ATRA and 9-cis-RA induce RARβ mRNA and protein in the 1483 cells, a process known to be mediated by activation of a DR-5 RARE by RXR-RXR heterodimers (35).

We found that 9-cis-RA activated transcription of reporter gene driven by a promoter containing the DR-5 but not the DR-1 response element (Fig. 1A). The lack of RXRE activation observed in other cell types, for example keratinocytes (44), may be due to low endogenous RXR-RXR levels and transrepression by RXR-RXR heterodimers that bind to the DR-1 with a higher affinity than RXR-RXR homodimers do but fail to activate this response element (16, 44). Therefore, the relative abundance of RXR and RAR may determine whether the RXR-RXR signaling pathway can be activated by 9-cis-RA (8, 9, 14, 15). One way to increase RXR levels is to introduce an exogenous receptor expression vector by transfection. Indeed, overexpression of RXRα in human keratinocytes enabled RXR-selective SR11237 to activate the RXRE via RXR-RXR homodimers (44). Unfortunately, such an approach has pitfalls.

**DISCUSSION**

amino acid 415–435 region in the human RXRα C terminus was critical for both homo- and heterodimerization as indicated by specific point mutations (37). To determine whether such mutations affect the ability of the receptor to mediate the growth inhibitory effects of 9-cis-RA in human HNSCC 1483 cells, we inserted each of three mutants, L418F, L430Q, and K431Q (Fig. 5A), into the pMark vector and analyzed their functions after transfection into the 1483 cells. Mutant L430F had lost all of the DR-1 activation potential (Fig. 5B2) and all of the ability to mediate inhibition of DNA synthesis by 9-cis-RA (Fig. 5B3) but still possessed about 50% of the DR-5 activation potential of wild-type RXRα (Fig. 5B1). Because the DR-5 activity in L430F-transfected cells was lower than in the pMark vector alone transfected cells, it seems that L430F interfered with the function of the endogenous RXR-RAR-mediated transcription via DR-5. The RXR mutant L430F retained approximately 80 and 90% of DR-1 and DR-5 activation potential of wild-type RXR, respectively, and 70% of growth inhibition. The activities of the RXR mutant K431Q were almost identical to those of the wild-type RXRα. Thus, the findings with the mutants indicate that growth inhibition by RXRα can be modified when its DR-1 activation by homodimer formation is compromised. To a lesser extent, this is also true for modulation of DR-5 transactivation by the RXRα mutants.

The RXRα Mutant F313A Activates Transcription from DR-1 and Suppresses DNA Synthesis in 1483 Cells in a Ligand-independent Fashion—9-cis-RA was required for transcriptional activation via DR-1 and for growth inhibition by RXRα and its active mutants L418F and K431Q. A mouse RXRα mutation (F318A) was reported to produce a constitutively active RXR by mimicking ligand-induced conformational changes. This mutant was able to activate DR-1 in the absence of ligand in Cos-1 cells (36). We prepared the human homolog F313A (Fig. 5A) and cloned it into the pMark vector to determine its effects on 1483 cells. Transient transfection of pMark RXRαF313A into 1483 cells increased transcriptional activity via the DR-5 in the absence of ligand by 6-fold relative to cells transfected with vector only (Fig. 5B1). Ligand-independent DR-5 activation was 66% of that observed in vector only transfected cells treated with 9-cis-RA. Thus, the mutant RXRα F313A can act as a functional partner for endogenous RARs in the absence of an RXR ligand. DR-5 reporter gene activation in 9-cis-RA-treated F313A-transfected cells was 1.7 times higher than in the absence of ligand. This increase can be due to the additive effect of the endogenous RXRα and the exogenous mutant F313A (Fig. 5B1). The activation of the DR-1 reporter gene in cells transfected with F313A was similar (about 10-fold) in the absence or presence of 9-cis-RA and similar to that of transfected wild-type RXRα in the presence of 9-cis-RA (Fig. 5B2). These results suggest that F313A forms constitutively active homodimers in 1483 cells. Transient expression of F313A in the absence or presence of ligand resulted in about 70% suppression of DNA synthesis (Fig. 5B3), which was similar to that obtained in cells transfected with exogenous wild-type RXRα and treated with 9-cis-RA. These results suggest that RXRαF313A homodimers can inhibit growth by mimicking the function of liganded wild-type RXRα homodimers.

**Fig. 5. Comparison of activation of retinoid response elements and suppression of DNA synthesis by point mutants and wild-type (WT) RXRα.** A, schematic representation of the RXRα point mutants cloned into the pMark vector. B, effects of these mutations on the transcriptional activity and growth suppression function of RXRα. Cells were transiently transfected with reporter constructs and with pMark vector or pMark into which wild-type or mutant RXRα was cloned. The cells were then treated with MeSO (DMSO) or 9-cis-RA. Transactivation of DR-5 (B1) and DR-1 (B2) and BrdU labeling index (B3) were determined as described under "Experimental Procedures."
Selection of the transfectants during a prolonged subculture and cloning may result in an altered phenotype particularly if RXRα overexpression causes growth suppression. The selection process produces cells refractory to growth inhibition by RXRα or cells that express low levels of receptor. To avoid such complications, we employed the co-expression vector pMark (31) to enable the simultaneous transient transfection of RXRα and analysis of growth inhibition of the transfected cells by a single cell assay based on BrdU incorporation into replicating DNA.

The transient expression of exogenous wild-type RXRα in 1483 cells resulted in 9-cis-RA-mediated activation of DR-1 as indicated by enhancement of the luciferase reporter gene transcription (Fig. 2A). The increased RXRα levels could have led to the formation of RXR homotetramers, which having a higher affinity for DR-1 than RXR-RAR may have displaced RXR-RAR heterodimers from the DR-1 RXRE (11). In the presence of 9-cis-RA, the DNA-bound tetramers may have dissociated to dimers (11) that activated DR-1-mediated transcription of the reporter. The expression of exogenous RXRα also modestly increased DR-5 activity by 9-cis-RA, possibly by increasing functional RXR-RAR heterodimer levels.

The DNA synthesis in 1483 cells was only minimally suppressed by 9-cis-RA despite the ability of this panagonist ligand (binding and activating both RARs and RXRs) to activate the DR-5. The activation of the DR-5 in the absence of an increase in DR-1 activity was observed in cells treated with RAR-selective TTNPB. This retinoid failed to suppress DNA synthesis (Fig. 3) and activate the DR-1. These results suggest that DR-5 activation is not sufficient to mediate growth inhibition in the parental 1483 cells and their RXRα transfecteds. HNSCC 1483 cells expressing exogenous RXRα showed an increase in the activation of both DR-5 and DR-1 response elements by 1 μM 9-cis-RA and also showed a decrease in DNA synthesis (Figs. 2 and 3). The four RXR-selective retinoids showed a tight correlation between their ability to activate DR-1 and to inhibit DNA synthesis in RXRα-transfected cells. The relationship between DR-1 activation and DNA synthesis suppression was further supported by the findings using RXRα mutants. Specifically, deletions in RXRα (e.g., RXRαΔΔ and RXRαΔF) and the point mutation L430F abolished DR-1 activation and suppressed inhibition of DNA synthesis by 9-cis-RA, although some ability to activate DR-5 was retained (Figs. 4 and 5). Only a few reports have shown that transfection of RXRα enhanced the retinoid response. For example, RXRα overexpression in HL-60 leukemia cells increased apoptosis, whereas transfection of RARα mediated induction of differentiation (45).

The mutation F318A in the ligand-binding pocket of mouse RXRα was found to cause the receptor to assume a conformation similar to that of agonist-bound wild-type RXRα and exhibit constitutive activation of the DR-1 (as a homodimer) and, to a lesser extent, a DR-5 reporter (as a heterodimer with RARs) in the absence of an RXR ligand (36). Although transcriptional regulation by this mutant has been characterized to a lesser extent, a DR-5 reporter (as a heterodimer with RARα) failed to induce differentiation of HL-60 leukemia cells and NTERa-2 teratocarcinoma cells, whereas VP16-RARα promoted the ligand-independent differentiation of both (47). These results are consistent with the observations that RXR-RAR pathway is dominant in mediating differentiation programs. The authors suggested that the chimeric constitutive receptors may be used as tumor suppressor genes for genetically based treatment of retinoid-responsive cancers (47). However, their data did not support the similar use of the RXR-specific signaling pathway. In contrast, we found that overexpression of RXRαF313A inhibited DNA synthesis. Our novel observation suggests that the RXR-RXR pathway may mediate growth inhibition that is possibly unrelated to induction of differentiation and that RXR overexpression can also be used to design new gene therapies.

It is not clear how overexpression of RXRα followed by 9-cis-RA treatment or expression of the constitutively active F313A causes inhibition of DNA synthesis in 1483 cells. Presumably, the overexpressed RXRα forms tetramers or dimers that are activated by RXR-selective ligands or by an activating mutation (i.e., F313A) to increase the transcription of genes that possess DR-1 RXREs, which are involved in regulation of cell growth by controlling DNA synthesis. In addition, overexpression of RXRα may lead to the formation of tetramers that could displace RXR-RAR heterodimers from natural DR-1 RXREs and thereby relieve the silencing of gene transcription. The identification of genes that are regulated by RXR homodimers may lead to the understanding of the mechanism by which RXR homodimers suppress growth. To date, only a few genes were shown to be regulated by RXR-selective retinoids, presumably via RXR binding to natural RXREs. Such genes include growth hormone in pituitary cells (41), α-fetoprotein in hepatocytes (42), and cholesterol 7α-hydroxylase in HepG2 cells (43).

Future studies will be necessary to determine whether RXR homodimers can directly regulate any genes involved in cell growth.

In conclusion, the results of several experimental approaches indicate that activation of the DR-1 RXRE is associated with ability of 1483 cells expressing exogenous RXRα to respond to 9-cis-RA with diminished DNA synthesis. Similar effects were also observed in four of five other human head and neck and lung cancer cell lines (data not shown). Thus, it may be possible to develop several therapeutic strategies based on our findings (for example, treatment with RXR-selective retinoids that activate endogenous RXRs to form RXR-RXR homodimers rather than heterodimers). Such an approach may be possible because the conformational changes in RXR required for homodimerization and heterodimerization can be separately modified (36, 37). Such novel retinoids could retain the minimal side effects characteristic of the currently available RXR-selective retinoids compared with the more deleterious RAR-selective retinoids (25, 48). Another strategy could be to activate the RXR-RXR pathway using gene therapy based on transfer of the RXRα gene followed by treatment with an RXR-selective retinoid or gene transfer of the constitutively activated RXRαF313A without the need to treat with a retinoid.

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