The divergent response and the molecular mechanisms underlying the anti-cancer effects of retinoid X receptor (RXR) ligand (rexinoid) therapy are poorly understood. This study demonstrates that ligand-activated RXR homodimer facilitates G1 arrest by up-regulation of p21 in vitro but failed to induce G1 arrest when p21 expression was blocked by p21 small interfering RNA. RXR ligand-dependent p21 up-regulation was transcriptionally controlled through the direct binding of RXR homodimers to two consecutive retinoid X response elements in the p21 promoter. Structural overlap of a retinoic acid response element with these retinoid X response elements led to a high affinity binding of retinoic acid receptor/RXR heterodimer to the retinoic acid response element, resulting in the prevention of RXR ligand-mediated p21 transactivation. These data show that p21 is a potential and novel molecular target for RXR ligand-mediated anti-cancer therapy and that the expression level of retinoic acid receptor and RXR in tumors may be crucial to induce p21-mediated cell growth arrest in RXR ligand therapy.

Retinoids are natural and synthetic vitamin A derivatives that regulate a multitude of biological processes in mammalian cells, including metabolism, development, cell proliferation, differentiation, and carcinogenesis (1). Carcinogen exposure causes squamous metaplasia of tracheal and other epithelia, and administration of vitamin A prevents tumor formation (2). Retinoids are clinically used to target selected human cancers (3, 27). The actions of retinoids are mediated by two distinct classes of retinoid receptors, retinoid acid receptors (RARβ, -β, and -γ) and retinoid X receptors (RXRα, RXRβ, and RXRγ), which require all-trans-retinoic acid and 9-cis-retinoic acid, respectively, for transcriptional activation. RXR is a combinatorial partner in the nuclear receptor family that forms homo- or heterodimers with a variety of hormone and orphan receptors, including RAR, vitamin D receptor, thyroid hormone receptor (TR), peroxisome proliferator-activated receptor (PPAR), chicken ovalbumin upstream promoter transcription factor (COUP-TF), and other receptors (4). The dimeric receptors bind to specific DNA response element of target genes, and this DNA binding specificity is determined by the number of spacer nucleotides present between the two direct repeats of the canonical binding sequence AGGTCA (5). For instance, RAR and RXR heterodimer binds to retinoic acid response element (RARE) that contains two or five spacers (DR2 or -5), and RXR homodimer binds to retinoid X response element (RXRE) in which the half-sites are separated by a single nucleotide (DR1) (5). Direct binding of ligands to the receptors is required for transcriptional activation, and in the absence of ligands, this activation is blocked by binding of specific repressors to the receptors (6). Ligand binding triggers a release of the repressors from the complex and promotes interaction with co-activator complexes that forms a bridge between the nuclear receptors and the basic transcriptional machinery (6). This tight regulatory control of the ligand-dependent transactivation of the nuclear receptor superfamily potentiates a multitude of therapeutic applications for anti-cancer treatments. However, clinical application of RAR ligand therapy has only shown limited efficacy possibly due to 1) multiple adverse effects caused by the requirement of large dosage to reach therapeutic efficacy and 2) frequent loss of, or reduced RAR expression in various cancer types. Conversely, RXR ligands (rexinoid) have shown promising chemopreventive and chemotherapeutic activities with mild toxicity in several cancer types tested (7), and RXR expression is rarely lost in human tumors (8). Administration of RXR ligand effectively suppressed tumor development in carcinogen-induced and transgenic mouse breast cancer models (9). RXR ligand therapy in cutaneous T cell lymphoma clinical trials showed anti-tumor activities with moderate toxicity (7), and
p21\textsuperscript{WAF1/CIP1} Is a Novel Molecular Target of RXR

non-small cell lung cancer patients in phase I and II clinical trials exhibited disease stabilization and improved survival with RXR ligand treatment (10). Despite these antitumor actions of RXR ligand, the molecular mechanisms underlying their efficacy are still poorly understood.

Conditional RXR knock out studies suggested biological roles of RXR in adult tissues, further highlighting the relevance of RXR signaling pathways to cancer. Ablation of RXRα causes epidermal interfollicular hyperplasia, keratinocyte hyperproliferation, and aberrant terminal differentiation in skin (11) and multifocal hyperplasia in prostate epithelium (12). Likewise, a reduction of RXRα expression in basal cells occurs in the early stage of prostate carcinogenesis (13). RXRα-null F9 embryonal carcinoma cells are resistant to retinoid-mediated cellular differentiation, anti-proliferation, and apoptosis (14). Similarly, RXR ligand demonstrates growth inhibitory effects in multiple cancer cell lines (15–17). Together, these findings suggest a role for RXR in tumor pathogenesis and the potential use of RXR ligand in cancer therapy.

Cyclin-dependent kinases (cdks) and cdk inhibitors are the principle regulators of cell cycle progression, especially in the G1 to S phase transition checkpoints. The activity of cyclin-dependent kinase can be inhibited by two groups of cyclin-dependent kinase inhibitors. Of these, p21\textsuperscript{WAF1/CIP1} plays an essential role in growth arrest (18) and also directly inhibits DNA polymerase activity by binding to the PCNA subunit (19).

p21-null mice are more prone to spontaneous and carcinogen-induced tumorigenesis (20), and pathological studies demonstrate that p21 expression correlates with a high apoptotic index in tumor cells, consistent with a favorable outcome in breast cancer patients (21). It is known that p21 is primarily regulated by p53 (22) but also by p53-independent mechanisms. Because p53 abnormalities are reported in more than 50% of human cancers, identifying a p21 regulatory mechanism that is p53-independent may suggest a novel cancer therapeutic approach. We now report that p21 is likely regulated by RXRα homodimer and that RXR-mediated up-regulation of p21 causes a cell cycle arrest at G0/G1 in p53-null cancer cells, thus identifying a novel regulatory mechanism of the p21 signaling pathway.

MATERIALS AND METHODS

Ligands—9-cis-Retinoic acid, methyprene acid, and TTNPB were obtained from Biomol (Plymouth, PA). RXR-selective pan-agonist AGN 194204 was a kind gift from Allergan Co. Ltd. (Irvine, CA).

Cell Culture—MDA-MB-231 mammary gland cancer cell line, H1299, non-small cell lung cancer cell line (NSCLC), and COS-1 African green monkey kidney cell lines were purchased from ATCC (Manassas, VA). Calu6 NSCLC cell lines were a generous gift from Dr. Jonathan Wiest (NCI, National Institutes of Health). MB-231 cells and COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 1200 mg/ml of Geneticin for 10 days. To generate stable clones, single colonies were picked and subsequently subjected to immunoblot analysis to detect VP16 expression followed by reporter assays using a recombinant adenovirus encoding TRE-luciferase.

Construction of Reporter Plasmids and Reporter Assay—A series of p21 promoter deletion mutants were generated by PCR with sense primers containing Xhol: from −916 to +13, 5′-aggtatgcaaaaaaacac-3′, from −1597 to +55, 5′-aggagttgccagacgag-3′, from −1012 to +55, 5′-aagggtacagacgag-3′, from −651 to +55, 5′-atatataatgccagag-3′, and from −312 to +55, 5′-actctttctcaagag-3′), and an antisense primer containing HindIII, 5′-ccggtctcgcagcaat-3′, and ligated into pGL-3 (Promega). Short deletion of putative RXRE (Fig. 3C) was generated by using Gene Editor system (Promega). Integrity of each clone was confirmed by DNA sequencing. The plasmid containing the p21 promoter sequence was co-transfected with RXR expression vector and pCMV-B-gal (Promega) to COS-1 cells. Transfection efficiency was normalized by β-galactosidase, and the luciferase activities from the treated cells were measured as described by the manufacturer (Promega). The light intensity was measured by a luminometer TROPIX TR717 (PerkinElmer Life Science). Data represent the means of at least triplicate experiments.

Northern Blot—Northern blot analysis was performed as described previously (24) with p21 cdNA. p21 mRNA was visualized by a PhosphorImager (GE Healthcare) and normalized by glyceraldehyde-3-phosphate dehydrogenase.

EMSA—Nuclear proteins were prepared from MDA-MB-231 cells as described previously (24), and recombinant RXR and RAR were purchased from ProteinOne (Rockville, MD). Equal amounts of nuclear proteins (10 μg) were incubated with 20,000 cpm of appropriate oligonucleotide probes in binding buffer (20 mM HEPES (pH 7.9), 10 mM KCl, 8% glycerol, and 1 mM dithiothreitol) for 20 min at room temperature with or without RXR ligand (10−6 M). For supershift assay, 0.4 μl of RXR antibody (3A2, a kind gift from Dr. Pierre Chambon, INSERM, France) was incubated with the DNA-protein complex.

Gene Silencing Using siRNA—The cells were transfected with 100 nm concentrations of appropriate siRNA or nonspecific siRNA as a control. 5′-GACCAUGUGACAGUCAUCU-3′ siRNA (p21siRNA405) was used to knock down p21, and 5′-GAAUGUGUGUACAGUCAUGU-3′ siRNA (RARα + -γ siRNA354) was used to knock down RARα and RARγ.
Cell Cycle Analysis—Ethanol-fixed cells were incubated with 100 units of RNase at room temperature for 20 min and incubated in propidium iodide for 30 min at 4 °C in the dark. Cell cycle was analyzed on a fluorescence-activated cell sorter Vantage instrument (BD Biosciences).

Xenograft—5-week-old female BalbC nu/nu nude mice were maintained in a VAF-barrier facility, and all animal procedures were performed in accordance with the regulation in the National Institute of Health Guidelines. Five million MDA-MB-231 cells (300 μl of serum-free Dulbecco’s modified Eagle’s medium) were injected subcutaneously into the back of nude mice and allowed to establish tumors for 2–3 weeks (300 μl of serum-free Dulbecco’s modified Eagle’s medium) were injected subcutaneously into the back of nude mice and allowed to establish tumors for 2–3 weeks

Histological Analysis—BrdUrd (20 mg/ml) was injected to mice intraperitoneally 1 h before harvesting the tumors. Dissected tumors were fixed overnight in 10% buffered formalin and embedded in paraffin. For immunohistochemistry, the slides were deparaffinized and rehydrated, and antigen was retrieved at 100 °C for 2 min in pH 7.0. In vivo apoptosis was detected by Apoptag kit (Biovision). In situ cell proliferation assay was performed as described by the manufacturer (Roche Applied Science).

Statistical Analysis—Values are the means ± S.D. The difference between the means of the treatment groups and appropriate controls were assessed with the paired two tailed t test; difference were considered to be significant if p < 0.05.

Chromatin Immunoprecipitation Assay—A chromatin immunoprecipitation assay was performed by following the manufacturer’s instruction (Upstate). Shared chromatin was immunoprecipitated with the following antibodies; 2.5 μl of RXR, RAR (a kind gift from Dr. Pierre Chambon, INSERM, France), and 5 μl of PPAR (Cayman, CA). The same amount of IgG was used for control. The chromatins were reverse cross-linked and used for PCR with sense primers 5’-TGTTTCAGAT-GAACATCCAT-3’ and antisense primer 5’-TGGAAGTGCTACTGCTTTCTT-3’ spanning the p21 RXREs region.

RESULTS

Ligand-Dependent Activation of RXRα Induces G1 Cell Cycle Arrest—We previously reported that the highly malignant human breast cancer cell line MDA-MB-231 does not respond to RXR ligand due to altered localization of RXRα to the splicing factor compartment, thereby sequestering its functional activity as a nuclear receptor (23). Thus, MB-231 was used in this study as RXRα functional knock-out cells to investigate biological mechanisms of the anti-cancer effect of RXRα in vivo and in vitro. RXRα overexpression or RXR ligand treatment alone in MB-231 cells did not induce G0/G1 arrest (Fig. 1A). In contrast, the combination of RXRα overexpression and RXR ligand treatment (column 8) induced G0/G1 cell cycle arrest, increasing the G0/G1 population by 16% over Ad-null infected non-treated cells at 36 h (Fig. 1A). In the presence of RXR, RAR ligand showed no effect on cell cycle, suggesting the cell cycle arrest was essentially mediated by RXR ligand (data not shown). To elucidate the mechanism underlying RXR-mediated G0/G1 arrest in response to RXR ligand, we examined the expression of cell cycle markers that control G1/S transition. Immunoblots showed that RXR ligand treatment up-regulated p21 within 24 h, and hyperphosphorylated pRb was significantly reduced within 48 h (Fig. 1B). RXRα overexpression and ligand treatment did not alter expression of other G1 cell cycle regulators, including p15, p16, p27, and cyclin D. Cyclin E was reduced with RXRα overexpression, but this reduction was not ligand-dependent (Fig. 1B). RXR overexpression also induced p21 in a RXR ligand-dependent manner in MCF-7 breast cancer cells (supplemental data). Furthermore, RXR ligand treatment caused an increased amount of p21 and cyclin E cell cycle inhibitory complex in the presence of RXR, but the formation of p21 and cyclin D complex was not increased (Fig. 1C). RXR ligand also facilitated the formation of E2F-Rb complex, indicating that G1/S transition was blocked (Fig. 1C). Furthermore, p21 siRNA reduced the expression of p21 (Fig. 1E) and inhibited the RXRα-mediated G0/G1 arrest (Fig. 1D), indicating that p21 up-regulation is a primary biological response to RXR-mediated cell growth arrest.

RXR Ligand Regulates p21 Expression—A possible regulatory mechanism of the RXR-mediated p21 up-regulation was investigated under different experimental conditions by varying the type, duration, or concentration of ligand treatment and the amount of exogenous RXR expression. p21 expression increased within 2 h of RXR ligand treatment in a dose-dependent manner (Fig. 2Ai) at the physiological ligand concentration of 10−9 M and higher (Fig. 2Ai). Increase in p21 expression was also directly proportional to the multiplicity of infection (m.o.i.) for adenosovirus encoding RXRα (Fig. 2Aii). The ligands known to activate RXR homodimers (9cRA, AGN194204, SR11345, and methoprene acid) induced p21 expression (Fig. 2Aiv). The ligands that are specific for RXR heterodimer partners such as RAR and vitamin D receptor failed to induce p21, whereas PPAR ligand induced p21 expression slightly (Fig. 2Av). Consistent with the protein expression, Northern blot showed that p21 transcript was up-regulated within 2 h of RXR ligand treatment (Fig. 2B). To further verify whether RXR regulates p21 transcription, a reporter construct containing the 2.4-kb p21 promoter was co-transfected with RXRα expression vector into COS-1 cells and then treated with RXR ligand. p21 reporter assays demonstrated that RXR ligand in combination with RXR overexpression markedly increased p21 promoter activity when compared with controls or RXR overexpression alone (Fig. 2Ci). p21 promoter activity was also elevated to a similar extent in response to all the other RXR ligands (Fig. 2Cii). Expression of RXRα induced p21 promoter activity to a significantly higher level than the RXRβ and γ isoforms. This indicates that RXRα is the favored isotype for p21 transcription (Fig. 2Ciii). p21 promoter activities increased in a RXR ligand concentration-dependent fashion (Fig. 2Civ). RXR ligand-induced p21 promoter activation was increased in a RXR dose-dependent manner. However, the two phase responses may indicate a possible involvement of more than one dimer with...
different affinity for RXR ligand such as PPAR. In fact, PPAR ligand (wy14643) activates not only p21 protein expression (Fig. 2A) but also p21 promoter activity (supplemental data). Together, these data demonstrate that p21 transcription is specifically regulated by the ligand-activated RXR.

**p21 Promoter Contains RXRE**—To identify a possible RXR regulatory element in the human p21 promoter, a series of deletion mutants was generated and co-transfected with RXRα/H9251 expression vector into COS-1 cells. RXR-ligand dependent p21 promoter activity did not significantly change after deletion up to H110021.6 kb (Fig. 3A). However, the promoter activity was dramatically reduced when the deletion extended below H110021.0 kb, indicating that the region between H110021.6 and H110021.0 kb contains a cis-element that responds to RXR ligand (Fig. 3A). Based on a bioinformatics program (Transfac Version 7.0), two putative RXR binding motifs were found in the region 22 bp apart from each other (Fig. 3A). RXRE1 (H110021198 to H110021186, 5'-AGGTCAgGGGTGT-3') was highly homologous to the major histocompatibility complex type RXRE, and RXRE2 (−1221 to −1209, 5'-GAGGCAaAGGTGA-3') was 75% homologous to the canonical RXRE (Fig. 3B). Interestingly, both RXRE1 and RXRE2 sequences overlap with a previously reported RAR/RXR heterodimer binding sequence (RARE) (25). To determine whether these putative RXREs are direct transcriptional targets for RXRα/H9251, the RXRE sequences were deleted in multiple combinations. The deletion of both RXRE1 and RXRE2 abolished the ligand-mediated p21 promoter activity close to the baseline level, but deletion of RXRE1 or RXRE2 alone partially reduced the promoter activity (Fig. 3B). Thus, both RXRE1 and RXRE2 are functional elements for RXR signaling.

**RXRα Homodimer Binds to p21 RXREs**—To confirm whether RXRα binds to the RXRE sites of p21 promoter, EMSA was performed with three different 32P-labeled oligonucleotide probes as shown in Fig. 3C (RXREs/RARE probe contains the region spanning RXRE1, RXRE2, and RARE, RXRE1 probe spanning only RXRE1, and RXRE2 probes only spanning...
RXRE2). Nuclear protein extracts from the MB-231 cells infected with Ad-RXRα formed a greater amount of DNA-protein complex than the Ad-null-infected control (Fig. 3Cii). In agreement with the previous report (26), RXR ligand further facilitated the formation of DNA-protein complexes (Fig. 3Cii). The complex formation was completely abolished when an excess amount of specific non-radiolabeled oligonucleotide was added (Fig. 3Cii). To confirm RXRα binding to the RXRE probes, RXRα and RAR antibodies were used for a supershift assay. The DNA-protein complexes were supershifted by RXRα antibody but not by RARα antibody or pre-immune serum (Fig. 3Cii). Consistent biding properties were detected in all three different DNA probes. Bacterially expressed recombinant RXRα also formed a DNA-protein complex with all p21 RXREs, and these complex formations were increased when a greater amount of recombinant RXRα was added (Fig. 3Ciii), demonstrating that RXR homodimer binds to both RXRE1 and RXRE2. Notably RXR homodimer binding to RXREs/RARE resulted in the same migration as binding to RXRE1 and RXRE2 alone, suggesting that RXR homodimer may selectively bind to either RXRE1 or RXRE2. These data demonstrate that RXR homodimers bind to p21 RXREs and that the presence of RXR ligand significantly increases the DNA binding affinities of RXR. These data were also supported by a chromatin immunoprecipitation assay which showed that RXR ligand induced RXR binding to the p21 promoter region, indicating RXR homodimer binding to the RXREs. Notably, RAR also binds weakly to the p21 promoter, but this interaction was not RXR ligand-dependent (Fig. 3D).

**Ligand-mediated RXRα Activation Inhibits the Growth of MB-231-grafted Tumors—**To assess the efficacy of RXRα as a therapeutic molecule in vivo, MB-231 cells were used to generate subcutaneous tumors in nude mice, and RXRα expression vector (0–300 ng) and treated with ligand (10⁻⁶ m) for 24 h (v). Fold induction was calculated by comparison of luciferase activity from the cells treated with ligand over untreated control, and -fold induction was further compared from RXR-transfected cells over that of empty vector-transfected cells.
p21\textsuperscript{WAF1/CIP1} Is a Novel Molecular Target of RXR

A

![Diagram showing the p21 promoter activity](image)

B

p21 RXREs/RARE sequence

| DR1 (RXRE1)          | DR1 (RXRE2)          |
|----------------------|----------------------|
| cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga | cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga |

RARE (DR5)

-2400

-1014

| a | b | c | d | e | f |
|---|---|---|---|---|---|
| ggtca | gttcaggggtgt | aggggtgt | gaggcaagtacaggggagtgtgtaga | gaggcaagtacaggggagtgtgtaga | gaggcaagtacaggggagtgtgtaga |

p21 promoter activity (fold induction by ligand)

C

(i)

RXREs/RARE

RXRE 1
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga

RXRE 2
ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca
ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca

ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca

ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca

ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca

ttcagggcaaggggaaaggtgtcgaggggtgaggtgtagaaccacatca

RARE del RXRE
cagaaaggggaagaagtggaagttcaggggaggtgaggtgtaga

(ii)

| RXREs/RARE | RXRE 1 | RXRE 2 |
|------------|--------|--------|
| Prot       | N      | N      |
| 9cRA       | +      | +      |
| competitor antibody | X50 ns | X50 ns |
| antibody   | X      | A      |
| pi         | X      | A      |

(iii)

RXRα

RXREs/RARE

RXRE 1

RXRE 2

D

| IP | RXR | RAR | IgG | input |
|----|-----|-----|-----|-------|
| -  | +   | -   | -   | 172bp |

| ligand | p21 |
|--------|-----|
| -      | -   |
RXRα in Ad-injected tumors was detected within the proximity of the needle track as designated by N (Fig. 4A, lower panel). RXR ligand administration to the mice that received Ad-null caused reduction in tumor size by ~20% when compared with sesame oil (Fig. 4B). The inhibitory effect on tumor growth was up to 60% with a combination of RXRα and RXR ligand, suggesting that the ligand-mediated RXR activation maximized the anti-tumor activity (Fig. 4B). Histological analysis showed that Ad-RXRα and subsequent RXR ligand administration reduced the number of BrdUrd-positive cells by 70% (Fig. 4Ci) and increased the number of terminal dUTP nick-end labeling positive cells significantly (Fig. 4Cii) when compared with the tumors injected with Ad-null or Ad-RXRα treated with sesame oil. In contrast, immunoblot analysis of these tumor lysates confirmed that p21 protein expression was 2.5-fold higher in the tumors treated with Ad-RXRα and RXR ligand than in controls (Fig. 4D).

RAR Inhibits RXR Homodimer-mediated p21 Transactivation—The overlap of RARE with two RXREs in the p21 promoter raised the question as to whether RAR/RXR heterodimer binds to the RARE and interferes with the binding of RXR/RXR homodimers to the RXREs, in turn promoting a loss of RXR ligand-mediated p21 transactivation. RXR overexpression induced p21 promoter activity in the presence of RXR ligand (Fig. 5A, column 4 and 6); however, this RXR ligand-dependent p21 activation was reduced when RAR was co-expressed (Fig. 5A, column 10). RAR overexpression also induced p21 promoter activation in response to RXR ligand (Fig. 5A, column 8, 9, 11, and 12) but minimal activation in response to RXR ligand (Fig. 5A, column 7). This suggests that RAR/RXR heterodimer formation may inhibit RXR homodimer-mediated p21 transactivation. This RAR-mediated inhibitory effect was abrogated when RARE/DR5 was changed to RARE/DR3 (Fig. 5B), suggesting that RAR/RXR binding to the RARE regulates RXR homodimer-mediated p21 transactivation. To verify this RAR/RXR binding, a similar experiment was conducted by EMSA with a fixed amount of recombinant RXR and increasing amounts of recombinant RAR (Fig. 5C). RXR homodimer bound to p21 RXRE but did not bind to RARE (Fig. 5C) and increased amounts of RAR abolished RXR homodimer binding (Fig. 5C), suggesting that RAR/RXR heterodimer preferentially bound to RARE and inhibited RXR homodimer binding to RXRE. As an independent approach, Tet-On MB-231 cells were co-injected with cytomegalovirus-driven Ad-RXRα (limited expression) and cTAg-driven Ad-TRE-RAR (inducible expression), and the infected cells were treated with or without doxycycline. Expression of RAR induced by doxycycline reached the maximum level within 24 h (Fig. 5D), and subsequent RXR ligand treatment caused up-regulation of p21 in the cells infected with either Ad-RXR alone or with a combination of Ad-RXRα and Ad-TRE-RAR without doxycycline (Fig. 5D). However, the induction of RAR by doxycycline abrogated RXR ligand-mediated p21 up-regulation (Fig. 5D). Together, these data suggest that RAR is a potential repressor of RXR/RXR homodimer-mediated p21 activation.

RAR siRNA Sensitized RXR Ligand-mediated p21 Activation—An RNA-mediated interference approach was used to investigate RAR interference with the RXR-mediated p21 activation. RAR siRNA efficiently knocked down the expression of RARα and RARγ in MB-231 cells (Fig. 6A). It has been reported that liganded retinoid receptors are degraded through the ubiquitination pathway as a result of transcriptional activation, as observed in scrambled siRNA transfection (24). When the expression of these RARs was reduced, RXR ligand treatment alone up-regulated p21 expression even without RXRα overexpression (Ad-null), and RXRα overexpression (Ad-RXRα) significantly enhanced RXR ligand-dependent p21 up-regulation (Fig. 6A). The same experiment was conducted to measure possible alterations in cell cycle. The S phase population was slightly reduced with RAR siRNA in Ad-null infected cells, and this reduction was greater when RXRα was overexpressed (Fig. 6B), suggesting that RAR plays an inhibitory role in RXR-mediated G1/G0 cell cycle arrest. To verify whether the same regulation is present in other cancer cells, we tested NSCLC cell lines. RAR siRNA sensitized the response to RXR ligand and up-regulated p21 expression in two p53-null cell lines, NSCLC H1299 and Calu6, when compared with control siRNA (Fig. 6C). These data suggest that RAR is a common repressor that regulates the RXR ligand-mediated p21 activation in p53 null cancer cell lines.

DISCUSSION

Retinoid receptors play essential roles in the control of cell growth, differentiation, and apoptosis. Retinoids are successfully used for ligand therapy on selected types of tumors (27). However, despite some success with retinoid therapies, the mechanism by which retinoids control cell growth are not fully understood. A recent clinical trial with RXR ligand as a single therapeutic regimen has shown a favorable clinical outcome with mild toxicity for a selected subgroup of advanced lung cancer patients (7), suggesting that the downstream target of RXR would provide a mechanistic understanding of the anticancer effect of RXR ligands. We now report that p21 is a direct downstream target of the RXR ligand-activated retinoid recep-

FIGURE 3. Identification of the response element of RXR ligand-dependent p21 activation. A, p21 reporter assay with a series of p21 deletion mutants. Full-length and a series of p21 promoter deletion mutants were transfected with RXRα expression vector into COS-1 cells and treated with RXR ligand for 24 h. B, p21 reporter assay with a short deletion of RXREs. A short deletion spanning either RXRE1, RXRE2, or both was co-transfected with RXRα expression vector into COS-1 cells and treated with RXR ligand for 24 h. -Fold induction was calculated by comparison of luciferase activity from the cells treated with ligand over untreated control. Transfection efficiency was normalized by β-galactosidase. C, EMSA analysis with RXREs. i, oligonucleotide sequences for EMSA. ii, binding of nuclear extracts from MB-231 infected with Ad-RXRα (X) or Ad-null (N) to p21 RXREs was analyzed. The reaction mixture was incubated with 9cRA (at final concentration 10^-6 M) or vehicle for 20 min at room temperature. To test binding specificity, a 50-fold excess amount of nonspecific (ns) or specific cold oligoprobe (>50) was incubated with the reaction mixture. For the supershift assay, 0.4 μl of antibody against either RXRα (X), RARα (A), or pre-immune serum (pi) was added to the DNA/protein complex. iii, recombinant RXRα binding to RXREs. Increasing amounts of recombinant RXRα were incubated with three different RXRE oligo probes. The open arrow represents supershifted band, and solid arrow represents RXR homodimers. D, chromatin immunoprecipitation (IP) assay with p21 RXREs. MB-231 cells were pretreated with either Me2SO or 10^-6 M RXR ligand. The chromatin were immunoprecipitated with antibodies as indicated.
tor pathway in vivo and in vitro, and RARE adjacent to RXRE in the p21 promoter results in a divergent response to RXR ligand-induced p21 expression and G1 arrest.

Transcriptional activation through RXR homodimer is mediated by the DNA response element (RXRE). The RXRE sequence has been reported in genes including cellular retinol binding protein II, hepatitis B virus enhancer, major histocompatibility complex (MHC) class I, α-fetoprotein, and human RXRγ2 (28). One of the central questions of the RXR role was whether RXR homodimer functions as a transcrip-
tion factor in vivo, aside from its characterized role as a heterodimerization partner for other nuclear receptors. Zhang et al. (26) reported that RXR ligand is an essential requirement for the RXR homodimerization. However, endogenous RXR ligand (9cRA) level is almost undetectable under normal physiological conditions in the adult (29), suggesting that the amount of endogenous RXR ligand is not sufficient to facilitate RXR homodimer formation. When exogenous 9cRA was orally administered in adult rats, it was widely distributed and retained at high concentrations in various tissues (30). This
suggests that RXR/RXR homodimer may become active under the condition where successive amounts of exogenous ligand are given. Although the major binding site for RAR/RXR heterodimers is RARE, RAR/RXR also binds to RXRE albeit with lower affinity; nevertheless, this binding is significantly higher than RXR/RXR homodimers binding to RXRE and preventing RXR homodimer-mediated transcription from RXREs (31). These findings propose that RXR homodimer-dependent transcription may not occur in vivo due to the dominant-negative effect of favorable RAR/RXR heterodimer bindings to RXRE. However, this dominant-negative effect may vary depending on RXR expression level, because RXR homodimer-mediated transcription was enhanced when the equilibrium between RAR and RXR was artificially altered by RXR overexpression (32). Histopathological studies have revealed aberrant retinoid receptor expression during tumor progression (3). Reports suggest that down-regulation of RARs during tumor progression coincides with loss of response to RAR ligand (33), and the loss of RARβ is commonly observed at the early stage of tumorigenesis in multiple cancers (3). Conversely, every RXR isotype is overexpressed in 66% of breast ductal carcinomas in situ lesions (8), and in particular RXRα up-regulation is associated with malignant transformation (34). Collectively, these data indicate that activation of RXR/RXR homodimer may require exogenous RXR-ligand and high RXR expression, and the aberrant expression profile of RAR and RXR in tumors may provide an advantage for RXR-ligand–induced cancer therapy.

Liu et al. (25) demonstrated RAR/RXR heterodimer binding to the RARE of the p21 promoter where we have found that it overlaps with two consecutive RXREs. This specific binding is evident by the fact that the disruption of RARE from DR5 to DR3 abrogates the inhibitory effect of RAR on RXR homodimer-mediated p21 activation (Fig. 5B). In addition to RAR/RXR heterodimer binding to RARE, its dominant-negative binding to the RXREs may be possible, as suggested by weak supershift by the use of antibody against RAR and the presence of RAR/RXR heterodimer formation (Fig. 5C, lane 12–15). Overall, RAR/RXR heterodimer prevents RXR homodimer-mediated p21 promoter activation. These two distinct inhibitory mechanisms seem to regulate p21 gene activation intrinsically through the pleiotropic cis-element, RXREs/RARE. Similar to our findings, there have been a few examples that two distinct classes of transcription factors can recognize a common regulatory sequence. The vitamin D response element in the human osteocalcin gene can function as a RARE, mediating cross-talk between vitamin D and vitamin A (35). In the lactoferrin gene, RARE overlaps with the estrogen response element, and this overlapping element mediates multi-hormonal sensitivity in transcriptional activation (36). The retinoid signaling pathway can be paradoxical, and the divergent responses from multiple cell types may be attributed to the loss of or reduced receptor expression (33), altered localization (23), or intricate transcriptional regulation (36). Our study suggests that the type of ligand may need to be carefully selected to archive therapy on the basis of the availability of receptors. Gene profiles or proteomics approach may be helpful for the screening.

RXR ligand activates multiple pathways of RXR homodimer and permissive heterodimers, including PPAR, LXR, and PXR. In contrast, non-permissive heterodimers (e.g. RAR, vitamin D receptor, and TR) cannot be activated by the RXR ligand. For example, PPAR is known to induce differentiation, apoptosis, and anti-inflammatory activity and is suggested to be one of the major targets of RXR ligand-mediated anti-tumor effect (7). A recent report suggests that PPAR/RXR heterodimers can also bind to RXRE (DR1) and activate transcription in response to PPAR ligand or RXR ligand (37). PPARα and its ligand treatment caused a slight induction of p21 (Fig. 2A,v) and p21 promoter activity, but this induction was not mediated through p21 RXREs (supplemental data). Our data suggest that RXR activated p21 transcription through RXR homodimer bindings to RXREs. However, at this point we are not able to rule out the possibility that unknown RXR permissive heterodimer may play a role in activating p21 in response to RXR ligand. Further extensive investigation is required to understand whether RXR homodimers play a role in vivo.

The role of p21 in response to activated p53 upon DNA damage is to induce cell cycle arrest and promote apoptosis. In the case of a low degree of DNA damage, however, p21 blocks p53-mediated apoptosis (38). Possible anti-apoptotic roles of p21 have been observed mostly in p53 wild type cells, indicating that p53 activity may be the key determinant factor on whether p21 functions as pro-apoptotic, anti-apoptotic, or as a cell cycle regulator. Similar to this, pathological analysis from NSCLC and ovarian cancer patients showed that the 5-year survival ratio of p21-positive and p53-negative patients have the most favorable prognosis over p21-negative and p53-positive patients or over both positive (39–41). Moreover, in vivo gene therapy approach with adenovirus p21 has shown promising therapeutic potential in the treatment of various cancer types (42). Considering that p53 is frequently mutated or functionally defective but its downstream target p21 is rarely mutated in many advanced human tumors (43), therapeutic strategy to directly activate endogenous p21 in a p53-independent fashion is a promising approach for cancer therapy.
tus may be a crucial parameter to consider for enhancing the efficacy of p21-targeted RXR ligand therapy via the pleiotropic retinoid response element RXREs/RARE.

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