Unique Anti-Activator Protein-1 Activity of Retinoic Acid Receptor β

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ABSTRACT

The anticancer effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are encoded by three distinct genes (α, β, and γ). Recent studies have demonstrated that RARβ plays a critical role in mediating anticancer effects of retinoids. However, how RARβ exerts its potent anticancer effects remains largely unknown. In this study, we investigated anti-Activator Protein-1 (AP-1) activity of RARβ. In a transient transfection assay, all three RAR subtypes, RARα, RARβ, and RARγ, could effectively inhibit phorbol ester 12-O-tetradecanoylphorbol-13-acetate-induced AP-1 activity and the activity of oncogenes c-Jun and c-Fos on AP-1 containing reporter genes in the presence of retinoic acid (RA). However, RARβ showed a strong RA-independent inhibition of AP-1 activity, whereas inhibition of AP-1 activity by RARα and RARγ was RA dependent. By using several hybrid receptors that contain either the COOH-terminal portion or the NH2-terminal portion of RARβ, we demonstrated that the NH2-terminal portion of RARβ, the A/B domain, was mainly responsible for the RA-independent inhibition of AP-1 activity. This activity was not attributable to constitutive AF-1 activity of RARβ, because it did not activate several RA response element-containing reporter genes. In addition, inhibition of histone deacetylase activity by trichostatin A did not overcome the inhibitory effect of RARβ. In cancer cells, stable transfection of RARβ exhibited strong inhibition of AP-1 activity, even in the absence of RA. Moreover, expression of endogenous AP-1-responsive gene collagenase I was strongly repressed in cancer cells stably transfected with RARβ. In studying the antitransforming activity of RARβ, we observed that the growth of breast cancer MDA-MB231 cells in soft agar was significantly repressed in a RA-independent manner when cells were stably transfected with RARβ but not RARα. Together, our results demonstrate that RARβ may exert its potent anticancer effect in part through its unique anti-AP-1 activity.

INTRODUCTION

Retinoids, a class of natural and synthetic vitamin A analogues, exert profound effects on many biological processes, including cell proliferation and differentiation, vision, reproduction, morphogenesis, and pattern formation, both in normal and transformed cells (1, 2). They have been well recognized as promising anticancer agents (3). The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RARs and RXRs (4–6). 9-cis-RA is a high affinity ligand for both RARs and RXRs, whereas all-trans-RA is a ligand for only RARs. RARs and RXRs are encoded by three distinct genes (α, β, and γ) and are members of the steroid/thyroid hormone receptor superfamily that function as ligand-activated transcription factors (4–6). RARs and RXRs primarily function as RXR/RAR heterodimers that bind to a variety of RAREs and regulate their transactivation activities (4–6). Regulation of gene expression by retinoid receptors requires interaction with additional cofactors that appears to provide a direct link to the core transcriptional machinery and to modulate chromatin structure (7).

In addition to their positive regulation of gene transcription, retinoid receptors also function as negative transcriptional factors (8). One of the well-known transcriptional repressive effects of retinoid receptors is their inhibition of AP-1 activity (8). Retinoid receptors, in response to their ligands, can inhibit the effect of tumor promoter TPA by repressing the transcriptional activity of AP-1 (9, 10). AP-1 is composed of proto-oncogenes c-Jun and c-Fos, the activity of which is often associated with cell proliferation and tumor progression (11). AP-1 activity is regulated by growth factors, cytokines, oncogenes, and tumor promoters that activate protein kinase C. It induces transcriptional activation by binding to TRE (11). The mechanism by which ligand-activated retinoid receptors repress AP-1 activity remains largely unknown, although a direct protein-protein interaction between retinoid receptors and AP-1 (9, 10) and a competition for a common coactivator (12) have been proposed. Nevertheless, the interaction between membrane and retinoid receptor signaling pathways may represent an important mechanism by which retinoids exert their potent antineoplastic effect (8). RA could prevent transformation of JB6 mouse epidermal cells promoted by TPA (13) and counteract the effect of TPA on expression of collagenase and stromelysin, presumably through inhibition of AP-1 activity (14). In addition, synthetic retinoids that selectively inhibit AP-1 activity and cannot induce transactivation of RA-responsive genes were able to inhibit the growth of lung and breast cancer cells (15, 16).

Recent studies have demonstrated that RARβ plays a critical role in mediating the anticancer effect of retinoids in many different types of cancer cells (17, 18), including breast cancer (19–21), lung cancer (22–27), ovarian cancer (28), cervical cancer (29), prostate cancer (30), neuroblastoma (31), renal cell carcinoma (32), pancreatic cancer (33), liver cancer (34), and head and neck cancer (35). Expression of RARβ in RARβ-negative cancer cells restored RA-induced growth inhibition, whereas inhibition of RARβ expression in RARβ-positive cancer cells abolished RA effects (19, 20). In addition, transgenic mice expressing RARβ antisense sequences showed increased incidence of lung tumor (25), whereas suppression of RARβ expression was responsible for diminished anticancer activities of retinoids in animal (36), and up-regulation of RARβ is associated with a positive clinical response to retinoids in patients with premalignant oral lesions (37). Furthermore, loss of RARβ was suggested to be an early event in carcinogenesis (26, 38–40) and may be involved in liver cancer development (41).

How RARβ exerts its anticancer effects remains largely unknown. We have demonstrated previously that expression of RARβ in certain breast cancer and lung cancer cells could induce apoptosis (20, 22), suggesting that apoptosis induction could contribute to the anticancer activities of RARβ. However, expression of RARβ does not always induce apoptosis of cancer cells (20, 22), indicating that mechanism other than apoptosis induction mediates anticancer effects of RARβ. In this study, we evaluated anti-AP-1 activity of RARβ. Unlike RARα and RARγ which repressed AP-1 activity in a RA-dependent manner, RARβ could repress TPA-

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3 The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; RAR, retinoic acid receptor response element; TPA, 12-O-tetradecanoylphorbol-13-acetate; AP, activator protein; TRE, TPA response element; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tk, thymidine kinase; TSA, trichostatin A.
induced AP-1 activity and activity of oncogenes c-Jun and c-Fos in a RA-independent manner. By using various hybrid receptors and RARβ deletion mutants, we demonstrated that the NH₂-terminal portion (A/B domain) of RARβ is mainly responsible for the RA-independent anti-AP-1 activity of RARβ. On several RAREs, RARβ did not show any constitutive AF-1 activity associated with the A/B domain. In addition, inhibition of histone deacetylase activity by TSA did not relieve the inhibitory effect of RARβ. These observations suggest that competition for common coactivator or the recruitment of receptor corepressor by RARβ is unlikely the mechanism for its effect. Furthermore, we found that cancer cells constitutively expressing RARβ, but not RARα, exhibited reduced AP-1 activity, even in the absence of RA treatment. Moreover, expression of endogenous AP-1 responsive gene
collagenase I and the growth of cancer cells in soft agar was significantly inhibited in a RA-independent manner in cancer cells stably expressing RARβ. Together, our results demonstrate that RARβ has a unique anti-AP-1 activity and that this unique RA-independent inhibition of AP-1 activity may represent one of the mechanisms by which RARβ exerts its potent anticancer activities.

MATERIALS AND METHODS

Cell Culture. HeLa and MDA-MB231 cells were grown in DMEM supplemented with 10% FCS. SK-MES-1 cells were maintained in MEM supplemented with 10% FCS. H292 cells were grown in RPMI 1640 supplemented with 10% FCS.

Plasmid Constructions. The CAT reporter constructs, -73Col-CAT, TRE-tk-CAT, and TREPal-tk-CAT, have been described previously (9, 42). Construction of expression vectors for RARα, RARβ, RARγ, c-Jun, and c-Fos and of RAR hybrid receptors were described previously (9, 42–44). RARβΔAB was constructed by cloning BamHI-flanked PCR product, using forward primer 5'-CGG GAT CCC GAA TGT ACA AAC CCT GCT TCG TC-3' and reverse T1 primer, into pcDNA3 vector. RARβΔE was generated by deleting the EcoRI fragment from the receptor.

Transient and Stable Transfection Assay. For HeLa cells, they were plated at 1 × 10^6 cells/well in a 24-well plate 16–24 h before transfection as described previously (43). For cancer cells, 5 × 10^4 cells were seeded in a six-well plate. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (42). Briefly, 250 ng of reporter plasmid, 100 ng of β-galactosidase expression vector (pCH 110; Pharmacia), and various amounts of expression vector were mixed with carrier DNA (pBluescript) to 1000 ng of total DNA/well. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. For stable transfection, the pCMV-RARβ recombinant plasmid was stably transfected into SK-MES-1, H292, and MDA-MB231 cells using the calcium phosphate precipitation method and screened using G418 (Life Technologies, Inc., Grand Island, NY) as described (20, 22). The integration and expression of transfected cDNA were determined by Southern blotting and Northern blotting, respectively. The stable clones obtained have been described (20, 22).

Multi-RT-PCR Assay. For multi-RT-PCR analysis, total RNAs were isolated and purified by Qiagen RNaseasy Mini kit. Expression of AP-1 responsive gene collagenase I in cells was determined by the multi-RT-PCR, modified according to the method described (45). Briefly, 1.5 μg total RNA were used for reverse transcription in 20 μl of reaction mixture containing 500 ng of oligo (dT)12–18, 250 μM deoxynucleotide triphosphates, first strand buffer, and 200 units of superscript II (Life Technologies, Inc.). PCR was carried out with collagenase I primers and GAPDH primers in one reaction system under optimized running conditions. PCR products were analyzed on 2.5% agarose gel and visualized by ethidium bromide staining. The primers used were as follows: collagenase I, 5'-ATGGGAGGAGCAAGAGAGGCTGGG-3' (sense); 5'-TTCCAGGTATTTTCTGGACTAAGTC-3', GAPDH, 5'-CCATCCACCATCTTTCCAGAGG-3' (sense), 5'-CCTGTTTCACCACACTTCTTG-3'.

Soft-Agar Assay. About 20,000 cells in culture medium containing 10% FCS, 0.3% agar (Difco, Detroit, Michigan), and 10^7 m all-trans RA in a six-well plate were plated onto an already hardened 0.6% agar layer under medium supplemented with 10% FCS. The plates were incubated for 21 days with 6% CO2. A colony was defined as >40 cells, and colonies with >40 cells were counted with a microscope.

RESULTS

RA-independent Inhibition of AP-1 Activity by RARβ. Inhibition of AP-1 activity by retinoid receptors is known to represent one of the mechanisms by which retinoid receptors exert their anticancer activity (8). Previous studies have demonstrated that RARβ exhibits tumor-suppressive effect and is a key retinoid receptor mediating anticancer activity of retinoids (17–35). To study the potent anticancer effect of RARβ, we evaluated its anti-AP-1 activity by transient transfection assay using a CAT reporter containing the collagenase promoter (~73Col-CAT), which is known to be activated by AP-1 activity (9). For comparison, the anti-AP-1 activity of RARα and RARγ was studied. The ~73Col-CAT was transiently transfected into HeLa cells together with each of RARs. Cells were then treated with or without 100 ng/ml TPA in the presence or absence of 10^-6 M RA. As shown in Fig. 1A, treatment of HeLa cells with TPA strongly induced the reporter transcription with about a 5-fold induction. Induction of the collagenase promoter activity by TPA was mainly attributable to its induction of the endogenous AP-1 activity (9). In the absence of cotransfected RAR expression vector, RA treatment slightly inhibited the TPA-induced reporter activity, with 27% inhibition, most likely attributable to activation of endogenous retinoid receptor. When we analyzed the effect of cotransfected RARs in the absence of RA, we observed a strong inhibition of the TPA-induced reporter activity when RARβ expression vector was cotransfected. The RA-independent inhibition of TPA activity by RARβ was concentration dependent. About 60% inhibition was observed when 50 ng of RARβ was cotransfected. RA treatment further enhanced the effect of RARβ. In contrast, inhibition of the TPA-induced promoter activity by RARα or RARγ required RA treatment. In the absence of RA, we did not observe any inhibition of the reporter activity when various concentrations of RARα or RARγ were transfected. These data demonstrated that RARβ could inhibit TPA activity in a RA-independent manner, whereas inhibition of TPA activity by RARα and RARγ requires their ligand binding. To determine whether the AP-1 binding site in the collagenase promoter was responsible for the observed effects, we used the TRE-tk-CAT reporter, in which the consensus AP-1 binding site (TRE) was linked with the tk promoter (42). About 6-fold induction of reporter transcription was observed when HeLa cells were treated with 100 ng/ml TPA (Fig. 1B). The TPA-induced TRE-tk-CAT reporter activity was significantly repressed when RARβ expression vector was cotransfected. In the absence of RA treatment, cotransfection of 50 ng of RARβ expression vector led to ~50% inhibition of TPA-induced TRE-tk-CAT activity. In contrast, repression of the reporter transcription by cotransfected RARα or RARγ occurred only when cells were treated with 10^-6 M RA. Together, these results demonstrate that RARβ could inhibit TPA activity in a RA-independent manner and that the inhibition is mediated by the AP-1 binding site.

To further determine the anti-AP-1 activity of RARβ, we studied its effect on activity of oncogenes c-Jun and c-Fos. Cotransfection of the TRE-tk-CAT reporter with c-Jun expression vector or c-Jun and c-Fos expression vectors in HeLa cells strongly induced the reporter gene transcription with a 4-fold (Fig. 1C) or a 5-fold (Fig. 1D) induction, respectively. Treatment of cells with RA only slightly inhibited the reporter transcription in the absence of RAR cotransfection. When RARβ expression vector was cotransfected, the reporter activity was strongly inhibited in a concentration-dependent manner. In contrast, when RARα or RARγ was cotransfected, the reporter activity was inhibited only when cells were treated with RA. Thus, expression of RARβ could repress transcriptional activity of oncogenes c-Jun and c-Fos in a ligand-independent manner.

RARs can heterodimerize with RXR and may function as a RAR/RXR heterodimer in cells (43). We therefore investigated whether expression of RXR had any effect on RA-independent inhibition of AP-1 activity. As shown in Fig. 1E, cotransfection of RXRα expression vector did not show any effect on TPA-induced ~73Col-CAT reporter activity. However, when RXRα was cotransfected with RARβ, RA-independent inhibitory effect of RARβ was enhanced. The enhancing effect of RXRα on RARβ activity was observed when three different concentrations of RARβ were used. The results therefore demonstrate that RARβ/RXR heterodimer could act as an effective inhibitor of AP-1 activity in the absence of RA.

To study whether the observed inhibition of AP-1 activity by
RARβ in the absence of RA treatment was attributable to a trace amount of endogenous retinooids, we evaluated the activation function of RARβ in response to various concentrations of RA by using the TREpal-tk-CAT reporter, which is known to be activated by RAR (43). For comparison, activation by RARα was analyzed. As shown in Fig. 1F, both RARα and RARβ showed a very similar response to various concentrations of RA in activating the TREpal-tk-CAT reporter. The maximum activation of the reporter by both receptors was observed when cells were treated with 10⁻⁶ M RA. These results demonstrate that activation of gene transcription by RARβ is RA dependent and suggest that repression of AP-1 activity by RARβ in the absence of exogenous RA is unlikely because of the presence of trace amounts of RA in the cells.

The A/B Domain of RARβ Is Responsible for RA-independent Repression of AP-1 Activity. To determine which domain of RARβ is responsible for its RA-independent anti-AP-1 activity, we analyzed the activity of several RARβ deletion mutants (Fig. 2A). Cotransfection of the −73Col-CAT reporter with a RARβ mutant deleted with the E domain (RARβΔE) significantly repressed the c-Jun-induced reporter activity in the absence of RA treatment (Fig. 2B). The observation is similar to that observed with the parental RARβ receptor (Fig. 1A), although the degree of inhibition by RARβΔE was reduced. Addition of RA treatment showed slight enhancement of the inhibitory effect by RARβΔE, which is likely attributable to the effect by endogenous RAR activity, because a similar degree of inhibition by RA was observed in the absence of cotransfected receptor. In contrast, cotransfection of a mutant lacking the A/B domain RARβΔAB did not show any inhibitory effect on c-Jun-induced reporter activity in the absence of RA (Fig. 2B), indicating that the A/B domain is crucial for RA-independent inhibition of AP-1 activity.

However, the mutant strongly inhibited the reporter activity when cells were treated with RA, demonstrating that the A/B domain is not required for RA-dependent inhibition of AP-1 activity. When a mutant deleted with the ABC domain (RARβΔABC) was analyzed, we found that both RA-dependent and -independent activities were abolished, indicating that the ligand-binding domain (E/F domain) alone is not sufficient to confer RA-dependent inhibition of AP-1 activity and that the C domain is also required. Together, these data demonstrate that the RA-independent anti-AP-1 activity of RARβ is likely mediated through the A/B domain of the receptor, whereas the RA-dependent activity requires both the DNA-binding domain and ligand-binding domain.

To further study the domain requirement of RARβ for its RA-independent anti-AP-1 activity, we constructed several hybrid receptors, in which the COOH-terminal portion or the NH₂-terminal portion of RARβ was replaced by the corresponding portion of either RARα or RARγ (Fig. 3A). Inhibition of AP-1 activity by these hybrid receptors was evaluated in HeLa cells using the −73Col-CAT reporter. As shown in Fig. 3B, cotransfection of hybrid receptors in which the COOH-terminal portion of RARβ was replaced with the corresponding portion from either RARα (RARβΔα) or RARγ (RARβγ) significantly inhibited TPA-induced reporter activity in a RA-independent manner, similar to that observed with the wild-type RARβ (Fig. 1A). In contrast, cotransfection of the hybrid receptor in which the NH₂-terminal domain of RARβ was replaced with RARγ (RARγβ) did not show any inhibitory effect on TPA-induced AP-1 activity in the absence of RA treatment. These data further demonstrate that the unique RA-independent anti-AP-1 activity of RARβ is mediated by the NH₂-terminal portion of RARβ.
RARβ Does Not Have Ligand-independent Transactivation Function. The mechanism by which nuclear receptors repress AP-1 activity is largely unknown. It was proposed recently that a competition of liganded retinoid receptors and AP-1 for their common coactivator, CREB-binding protein, may account for mutual antagonism between retinoid-activated receptors and AP-1 (12). To determine whether the RA-independent inhibition of AP-1 activity by RARβ was attributable to competition for the common coactivator by the A/B domain, which may possess a RA-independent transactivation function (AF-1), we evaluated three RAR subtypes for their RA-independent transactivation on several RAREs, including TREpal, βRARE, and CRBPI-RARE (27, 43), in HeLa cells (Fig. 4). Our results did not reveal any constitutive activation by RARβ on three RAREs examined. In contrast, RARγ that did not show any RA-independent inhibition of AP-1 activity (Fig. 1) displayed a significant RA-independent transactivation on these RAREs. The fact that RARβ lacks RA-independent transactivation function demonstrates that RA-independent inhibition of AP-1 activity by RARβ is unlikely attributable to its sequestration of a coactivator.

Inhibition of Histone Deacetylase Does Not Overcome the Inhibitory Effect of RARβ. Unliganded retinoid receptors are known to interact with nuclear receptor corepressors that form complexes with histone deacetylases (7). To gain insight into the possible mechanism of the ligand-independent repression of AP-1 activity by RARβ, we determined whether the inhibition was attributable to recruitment of the receptor corepressor by unliganded RARβ. We examined the effect of TSA, a specific inhibitor of histone deacetylases (46), on RA-independent inhibition of AP-1 activity by RARβ. As shown in Fig. 5, treatment of HeLa cells with TSA alone slightly enhanced the ~73Col-CAT reporter activity, probably because of inhibition of corepressor activity. TSA did not show a clear effect on TPA-induced reporter activity. When RARβ expression vector was cotransfected, the RA-independent inhibition of TPA-induced reporter activity was not affected by TSA treatment. These data suggest that histone deacetylase-associated activity is unlikely responsible for transcriptional repression of AP-1 activity by unliganded RARβ.

RA-independent Inhibition of AP-1 Activity in RARβ-negative Cancer Cells Stably Transfected with RARβ. We next evaluated anti-AP-1 activity of RARβ in SK-MES-1 lung cancer cells that do not express RARβ (47). We first examined anti-AP-1 activity of various RARs in this cell line by transient transfection assay using the TRE-tk-CAT reporter. When the reporter was transfected into SK-MES-1 cells, the reporter transcription was strongly induced by TPA treatment, with ~6-fold induction (Fig. 6). The TPA-induced reporter activity was slightly reduced when cells were treated with RA. When RARβ was cotransfected, we observed a RA-independent repression of the TPA-induced reporter activity in a concentration-dependent manner. Cotransfection of 100 ng of RARβ led to ~45% inhibition of
the TPA-induced activity. In contrast, RARα and RARγ did not exhibit any RA-independent repression of AP-1 activity in the cells. They repressed the TPA-induced activity only when cells were treated with RA.

We further determined the anti-AP-1 activity of RARβ in SK-MES-1 cells by stable transfection with RARβ. Two stable clones that expressed transfected RARβ (RARβ#6 and RARβ#7; Ref. 22) were evaluated for the ability of TPA to induce the TRE-tk-CAT reporter. As shown in Fig. 7A, TPA strongly induced transcription of the reporter gene in the parental SK-MES-1 cells, with ~5-fold induction. However, the ability of TPA to induce the reporter gene expression was completely suppressed in the stable clones. We did not observe any induction of the reporter transcription in SK-MES-1/RARβ#6 and SK-MES-1/RARβ#7 clones, either in the absence or presence of RA treatment. The reduced ability of TPA to induce reporter transcription was specific to RARβ transfection because expression of the empty vector (Vector) showed a similar response to TPA as compared with the parental cells. These data suggest that expression of RARβ could inhibit the ability of TPA to induce transcription of AP-1-responsive genes in a RA-independent manner.

The anti-AP-1 activity of RARβ was also evaluated in another RARβ-negative cancer cell line, MDA-MB231 breast cancer cells (20). MDA-MB231 cells only express low level of RARα (48). We therefore compared the effect of RARβ and RARα on the ability of c-Jun/c-Fos to induce −73Col-CAT reporter transcription in the absence or presence of RA. As shown in Fig. 7B, c-Jun/c-Fos strongly induced the reporter transcription in the wild-type MDA-MB231 cells, with about a 5-fold induction. A similar degree of induction by c-Jun/c-Fos was also observed in MDA-MB231 cells stably transfected with the empty vector (Vector). In both MDA-MB231 and MB231/vector cells, c-Jun/c-Fos-induced reporter activity was slightly inhibited when cells were treated with RA, probably because of the presence of endogenous RARs (20). When MDA-MB231 cells transfected with RARβ or RARα were analyzed, we observed that c-Jun/c-Fos could still induce the reporter gene activity in the RARα stable clones (RARα#2 and RARα#21). The degree of induction was similar to that observed in the wild-type cells or Vector cells. c-Jun/c-Fos-induced reporter activity, however, was strongly repressed when cells were treated with 10−6 M RA, demonstrating that the transfected RARα could repress c-Jun/c-Fos-induced AP-1 activity in a RA-dependent manner. In contrast, the ability of c-Jun/c-Fos to induce the reporter gene transcription was significantly reduced in RARβ stable clones (RARβ#2 and RARβ#3), with only a 2-fold induction. Treatment of the RARβ stable clones with RA further reduced the ability of c-Jun/c-Fos to induce reporter transcription. Thus, stably transfected RARβ, but not RARα, could prevent activation of AP-1-responsive genes by c-Jun/c-Fos in the absence of RA.

Inhibition of Collagenase I Expression by Stable Expression of RARβ. We next examined the effect of RARβ expression in SK-MES-1 cells on the ability of TPA to induce expression of endogenous collagenase I gene expression by RT-PCR. Expression of collagenase I is known to be induced by TPA through its activation of AP-1 that binds to a TRE present in the promoter (14). As shown in Fig. 8A, TPA strongly induced expression of collagenase I by 10-fold in SK-MES-1 cells, as reported previously (14). Treatment of cells with RA slightly repressed induction of collagenase I by TPA. For comparison, we did not observe any effect of TPA or RA on expression of the GAPDH gene. Similar results were observed in SK-MES-1 cells stably transfected with the empty vector (Vector). However, the ability of TPA to induce collagenase I expression was largely reduced in the RARβ stable clones (RARβ#6 and RARβ#7), with only ~3-fold induction. Treatment of the cells with RA completely repressed the ability of TPA to induce collagenase I expression. To further confirm the RA-independent anti-AP-1 effect of RARβ, we compared the inducibility of TPA on collagenase I expression in H292 lung cancer cells and H292 cells stably expressing transfected RARβ. Our result (Fig. 8B) showed that the ability of TPA to induce collagenase I expression was significantly reduced by overexpressing RARβ also in this cell line. These data further confirm that RARβ exerts a potent RA-independent anti-AP-1 activity.

Inhibition of Cancer Cell Growth by RARβ. Previous studies have demonstrated that expression of RARβ in RARβ-negative cancer cells could suppress growth of cancer cells (19, 20, 22, 49). To determine whether the RA-independent inhibition of AP-1 activity could contribute to its growth-inhibitory effect, we evaluated the effect of RARβ expression on growth of MDA-MB231 cells in soft agar (Fig. 9). The growth of the wild-type MDA-MB231 cells in the soft agar was not affected by RA treatment, as reported previously (20). However, the growth of MDA-MB231 cells expressing stably transfected RARβ (MB231/RARβ#3) was largely reduced, even in the absence of RA, with ~50% inhibition (Fig. 9). Treatment of
MB231/RARβ#3 cells with RA further inhibited the growth of the cells. In contrast, inhibition of the growth of MDA-MB231 cells expressing transfected RARα (MB231/RARα#2) was RA dependent. Our previous observation (20, 21) that RARβ was induced by RA in the MB231/RARα#2 cells suggests the RA-dependent inhibition of MB231/RARα#2 cell growth is in part mediated by the induced RARβ. The observed effect was specific because stable transfection of the empty vector (Vector) did not show any effect on growth of the cells. These results suggest that the potent and unique anti-AP-1 activity of RARβ may contribute to the anticancer activity of RARβ.

**DISCUSSION**

Retinoids have been shown as promising preventive and therapeutic agents for human cancers (3). The effects of retinoids are mainly mediated by RARs and RXRs (4–6). Both receptor types are encoded by three distinct genes (α, β, and γ). They exert their anticancer activities by modulating proliferation, differentiation, and apoptosis of cancer cells (1, 2). A growing literature has demonstrated that RARβ is primarily responsible for mediating the anticancer effect of retinoids (17–35). Here, we provide evidence that RARβ has an unique RA-independent anti-AP-1 activity that is different from other RAR subtypes, suggesting that RARβ may exert its potent anticancer effects in part through RA-independent inhibition of AP-1 activity.

The anticancer activity of retinoids is thought to be in part attributable to their direct antiproliferative effects, which have been observed in several transformed cell lines, including those for mammary, melanoma, lymphoid, and fibroblastic cancers (3). Studies in human breast cancer cells and bronchial epithelial cells also suggest that retinoids may inhibit proliferation through inhibition of transcription factor AP-1 activity (15, 50, 51), which is composed of c-Jun homodimers or c-Jun-Fos heterodimers. Several lines of evidence provided here demonstrate a unique RA-independent anti-AP-1 activity of RARβ. In transient transfection assays, cotransfection of RARβ or RARα and RXRα inhibited either TPA-induced AP-1 transcriptional activity or c-Jun-Fos transcriptional activity in a RA-independent manner, whereas inhibition of AP-1 transcriptional activity by RARα or RARγ was RA dependent (Fig. 1). The RA-independent repression of AP-1 transcriptional activity by RARβ was observed in several cell lines, including HeLa (Fig. 1), SK-MES-1 lung cancer (Figs. 6 and 7A), H292 lung cancer (Fig. 8B), and MDA-MB231 breast cancer (Fig. 7B) cells, suggesting that it may function in various cell types. The effect of RARβ could be also observed in RARβ-negative cancer cells stably transfected with RARβ. Stable expression of RARβ in RARβ-negative SK-MES-1 and MDA-MB231 cells reduced the ability of AP-1 to induce expression of AP-1-responsive genes, including the transactivated AP-1-responsive reporter genes (Fig. 7) and endogenous AP-1-responsive gene (Fig. 8).
Previous studies investigating domain requirement of AP-1/RAR interaction have shown that the ligand binding domain and DNA binding domain are required for AP-1/RAR interaction (9, 52). Consistent with these previous results, our data (Figs. 2 and 3) demonstrate that both the NH2-terminal portion (A/B and C domains) and COOH-terminal portion (E/F domain) of RARβ are involved in the inhibition of AP-1 transcriptional activity. In addition, by using various RARβ mutant receptors, we demonstrate that the A/B domain of the RARβ is responsible for the RA-independent repression of AP-1 transcriptional activity (Fig. 2). Cotransfection of the NH2-terminal half of the RARβ, but not the COOH-terminal half of RARβ, showed a RA-independent repression of AP-1 activity (Fig. 2). In addition, deletion of the A/B domain completely abolished the RA-independent inhibition of AP-1 transcriptional activity. Furthermore, hybrid receptors containing the NH2-terminal half of RARβ, but not RARα or RARγ, showed the RA-independent anti-AP-1 effects (Fig. 3). It is likely that the A/B domain of RARβ may directly interact with AP-1. Alternatively, the A/B domain of RARβ may interact with certain cofactors that mediate AP-1/RARβ interaction.

Recent results from our laboratory indicate that competition of liganded retinoid receptors and AP-1 for common transcriptional coactivator CBP may in part contribute to the mutual inhibition of their transcriptional activity (12). However, RARβ does not possess RA-independent transactivation function on several RAREs in HeLa cells (Fig. 4), indicating that sequestration of a coactivator is unlikely the mechanism for RA-independent inhibition of AP-1 activity. Unliganded retinoid receptor is known to interact with receptor corepressor (7). The fact that unliganded RARβ could repress AP-1 activity suggests that RARβ may inhibit AP-1 transcriptional activity through the recruitment of a receptor corepressor. Receptor corepressors are known to form a complex with histone deacetylases to mediate transcriptional repression (7). However, TSA, a specific inhibitor of histone deacetylase (46), failed to relieve the transcriptional repressive effect of unliganded RARβ (Fig. 5). This observation demonstrates that histone deacetylase-associated activity is unlikely the mechanism for RA-independent inhibition of AP-1 transcriptional activity by RARβ.

We have demonstrated previously that expression of RARβ could induce apoptosis of certain cancer cells in response to RA treatment (20). Our present finding suggests that inhibition of AP-1 activity may also contribute to the growth-inhibitory effect of RARβ. Thus, RARβ may exert its tumor-suppressive effect through different mechanisms, such as the anti-AP-1 effect and apoptosis induction, which are likely operated in a cell type-

Fig. 9. RA-independent inhibition of anchorage-independent growth of MDA-MB231 cells by RARβ expression. A, photograph of colonies formed by parental MDA-MB231, MB231/Vector, MDA-MB231 cells transfected with the empty vector; RARα (MB231/RARα#2) and RARβ (MB231/RARβ#3) in the absence or presence of all-trans RA (10⁻⁷ M). B, quantitation of colonies formed by MDA-MB231, vector, MB231/RARα#2, and MB231/RARβ#3 in the absence or presence of RA. Colonies were scored and expressed as percentages of the number of colonies formed by cells treated with solvent control.
dependent manner. Our observation that RARβ could inhibit AP-1 activity in the absence of RA may provide an explanation for the potent tumor-suppressive effect of RARβ. Thus, unlike other RAR subtypes, RARβ could act as an efficient tumor suppressor even in the absence of RA. This is clearly seen in our growth inhibition assay (Fig. 9), showing that expression of RARβ could repress the growth of MDA-MB231 cells in soft agar in a RA-independent manner whereas the growth-inhibitory effect of RARα is RA dependent. Previous studies have demonstrated that RARβ exerts tumor-suppressive effects in cancer cells (20, 22, and 49) and that a low expression level of RARβ may be an important contributing factor in cancer development (17–35). Our present finding suggests that the unique anti-AP-1 activity of RARβ may contribute to its tumor-suppressive effect. The mitogenic stimulus, often generated by the autocrine secretion of growth factors, is transmitted to cell nucleus to activate the nuclear transcriptional factors c-Jun and c-Fos, which often trigger cell proliferation. By inhibiting AP-1 transcriptional activity, RARβ may inhibit cell proliferation often associated with cancer development. Loss of this negative growth control mechanism likely plays a role in cancer development. Interestingly, overexpression of AP-1 could abrogate the growth-inhibitory effect of RA, resulting in retinoid resistance (51, 53). Thus, the cross-talk between AP-1 and retinoid receptor is reciprocal, and a balance between RARβ and AP-1 activity may contribute to the maintenance of the proper growth of cells.

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Unique Anti-Activator Protein-1 Activity of Retinoic Acid Receptor β

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