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Murine RARβ4 Displays Reduced Transactivation Activity, Lower Affinity for Retinoic Acid, and No Anti-AP1 Activity

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Abstract The biological actions of retinoic acid (RA) are mediated by retinoic acid receptors (RARα, β, and γ) and retinoid X receptors (RXR α, β, and γ). Each of the RARs is expressed as four to seven different isoforms. Four isoforms of RAR β (β1, β2, β3, and β4), which differ only in their N-terminal sequence (A domain) have been described. These RARβ isoforms display a specific pattern of expression in developing and adult animals and are highly evolutionarily conserved suggesting that they mediate distinct cellular effects of vitamin A. Experiments were performed to examine directly the RA-binding activity, transactivation activity, and anti-AP1 activity of each of these four RARβ isoforms. The results demonstrate that RARβ1, β2, and β3 bind RA with a similar K_d value, have a similar EC_{50} value in RA-dependent transactivation assays and inhibit AP1 activity to a similar level. By contrast, RARβ4 has an elevated K_d for RA, an increased EC_{50} value in RA-dependent transactivation assays and does not display the ability to inhibit AP1 activity. This provides additional evidence that at least one RAR isoform, RARβ4, may mediate distinct activities within a cell. Furthermore, these data suggest that the presence of an A domain in RARβ is important for modulating these activities of RARs. J. Cell. Biochem. 77:604–614, 2000.

Key words: retinoic acid receptors; isoforms; retinoic acid; anti-AP1; ligand binding; transactivation

Retinoic acid (RA), a vitamin A metabolite, and its synthetic analogues, are powerful regulators of cell proliferation and differentiation [for review, see Gudas et al., 1994]. These effects are mediated by two classes of ligand-dependent transcriptional regulators, which belong to the multigene family of steroid and thyroid hormone receptors, called retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [for review, see Chambon, 1996]. The RARs and RXRs are each composed of three receptor types, termed α, β, and γ [for review, see Chambon, 1996]. In vitro binding studies have demonstrated that the natural metabolites, all-*trans*-RA and 9-*cis*-RA, are high affinity ligands for RARs, whereas only 9-*cis*-RA has been shown to bind to RXRs [Heyman et al., 1992; Levin et al., 1992].

In dimeric form, RARs and RXRs function as ligand-inducible transcriptional regulatory proteins by binding to DNA sequences called retinoic acid-responsive elements (RARE) or retinoid X-responsive elements (RXRE) located in the promoter of target genes [for review, see Chambon, 1996]. Alternatively, they can indirectly inhibit the expression of genes by antagonizing AP1 (c-Jun/c-Fos)-mediated gene expression [Schule et al., 1991; Salbert et al., 1993]. The transactivation function and the AP1-antagonism functions of RARs can be separated pharmacologically using specific synthetic retinoids [Nagpal et al., 1995; Fanjul et al., 1994; Chen et al., 1995].

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RARs, like other members of the steroid and thyroid hormone superfamily, are composed of six structurally distinct domains (termed A–F), each of, which have been assigned specific functions [for review, see Chambon, 1996]. The N-terminal A and B domains contain ligand-independent transactivation activity (AF-1), while the highly conserved C domain contains two zinc fingers and is important for DNA binding and dimerization. The E domain is functionally complex, containing all the information necessary for high affinity ligand binding along with a ligand-dependent transactivation function (AF-2) and accessory dimerization sequences.

Each of the three RAR subtypes has a number of different isoforms. Murine RARα has two major isoforms, RARα1 and RARα2, and five minor isoforms [Leroy et al., 1991]; murine RARβ has four isoforms termed RARβ1, RARβ2, RARβ3, and RARβ4 [Zelent et al., 1991; Nagpal et al., 1992a]; and murine RARγ has two major isoforms, RARγ1 and RARγ2, and five minor isoforms [Kastner et al., 1990; Giguere et al., 1990]. Interestingly, only three isoforms of RARβ (RARβ1, RARβ2, and RARβ4 but not RARβ3) have been described in humans [Shen et al., 1991; Toulouse et al., 1996]. Each of the isoforms of a particular RAR subtype has identical B through F domains; however, the amino acid sequence of their A domains varies drastically both in size and amino acid composition. The RAR isoforms for each subtype arise from the use of different promoters and/or alternative splicing.

In the case of RARβ, two promoters are utilized along with alternative splicing to produce the different isoforms [Zelent et al., 1991; Nagpal et al., 1992a; Shen et al., 1991; Toulouse et al., 1996]. RARβ isoforms 2 and 4 arise from the same promoter called P2, which contains a strong RARE. The 5′ untranslated region of the RARβ4 transcript is spliced out including the ATG start codon. Therefore, RARβ4 translation is initiated from an internal CUG codon resulting in a RARβ isoform that lacks all but 4 of the A domain amino acids. In mice, RARβ1 and RARβ3 arise from a common promoter termed P1 and share a common 5′ untranslated region and N-terminal A domain whose amino acid sequence is different from that of RARβ2 and RARβ4. In murine RARβ1, there is a 81-bp-long exon (E3), which is spliced from the transcript. Therefore, the amino acid sequence of the murine RARβ3 differs from that of RARβ1 by additional 27-amino acid residues at the C-terminal end of the A domain. In humans, the P1 promoter gives rise to only one transcript, RARβ1, which displays no significant homology in the 5′ untranslated region of its mouse counterpart and appears to be expressed only in fetal tissues and certain tumors [Houle et al., 1994; Toulouse et al., 1996].

The function or importance of the different isoforms of RARβ is unknown. It has been suggested that the different A regions of the isoforms confer the functional specificity of the receptor, which in turn allows them to mediate distinct cellular effects during development and in the adult. This is supported by the observation that each of the RAR isoforms display a unique spatiotemporal pattern of expression in both the fetus and the adult mouse, and the A domain of each of the RAR isoforms is highly evolutionarily conserved [Zelent et al., 1991; Nagpal et al., 1992a; Kastner et al., 1990; Leroy et al., 1991]. As an initial step towards elucidating the unique function(s) of the four murine RARβ isoforms, we have compared their RA binding activity, transactivation activity and anti-AP1 activity. RARβ1, RARβ2 and RARβ3 displayed very similar activity in each of these assays, while RARβ4 had a 5- to 10-fold higher K_d for RA, a greatly elevated EC_{50} value in RA-dependent transactivation assays and no anti-AP1 activity. Since RARβ4 lacks all but 4 amino acid in its A domain, these results suggest that the A domain plays an important role in mediating ligand binding, transactivation activity and anti-AP1 activity of the RARβ isoforms.

MATERIALS AND METHODS

Plasmid Constructs

Mouse RARβ1, RARβ2, RARβ3, and RARβ4 cDNAs were cloned in the sense orientation into the Not*I* restriction site of the eukaryotic expression vector pOPRSVI/MCS (Stratagene), using the plasmid DNAs pSG5-RARβ2 and pBluescriptSK$^-$RARβ1,3 (both obtained as a generous gift from Professor Pierre Chambon, Institute de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France), and the polymerase chain reaction (PCR). The complete DNA sequence of each clone was confirmed by DNA sequence analysis [Sanger et al., 1977]. Each pET29a RARβ isoform prokaryotic expression clone was prepared by sub-

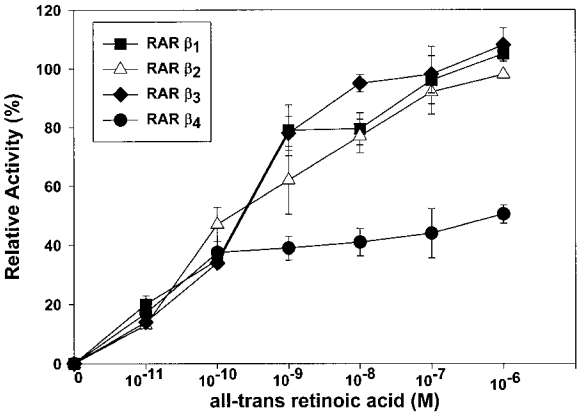

Fig. 1. Retinoic acid (RA)-dependent transactivation activity of the 4 RARβ isoforms. CV-1 cells were transfected with 0.01 μg of pOPRSV-RARβ isoform DNA, 1μg of CMV-βgal DNA and 3 μg of RARE-CAT reporter DNA. At 24 h after transfection, the cells were treated with the indicated concentrations of all-*trans*-RA or ethanol carrier. At 48 h after transfection, the cells were harvested and assayed for CAT and βgal activity. Percentage relative normalized CAT activity was calculated using relative CAT activity achieved with RARβ2 at 10^{-6} M all-*trans*-RA as 100%. Each data point represents the mean of at least three independent experiments performed in duplicate ±S.E. (bars).

cloning in frame the *Not*I fragment from the appropriate pOPRSVI construct. It should be noted that the CTG translation initiation codon of RARβ4 previously described by Nagpal et al. [1992a] was mutated to ATG in ensure efficient initiation of translation at this site.

Transactivation Assays

Transactivation assays were performed essentially as described previously [Tairis et al., 1994, 1995; Scafonas et al., 1997]. Briefly, CV-1 cells were plated at 400,000 cells/60-mm dish. The next day, the cells were transfected with 0.01 μg of one of the RARβ isoform expression constructs (pOPRSVI-RARβ1, pOPRSVI-RARβ2, pOPRSVI-RARβ3 or pOPRSVI-RARβ4), 3 μg of RARE-CAT reporter DNA obtained as a generous gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA) and 1 μg CMV-βgal DNA by the Ca^{2+} phosphate methodology. At 24 h later, the cells were treated with all-*trans*-RA ranging in concentration from 10^{-11} to 10^{-5} M prepared in ethanol or ethanol carrier alone. After an additional 24 h, the cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity [Seed et al., 1988] and β-galactosidase activity [Eustice et al., 1991]. CAT activity was normalized with respect to β-galactosidase activity to control for transfection efficiency and expressed as a percentage of the relative CAT activity. The fold induction in normalized CAT activity of RARβ2 at 10^{-6} M all-*trans*-RA compared with no RA treatment (ethanol control) was chosen as 100% relative activity. The EC_{50} value for each RARβ isoform represents the concentration of RA that resulted in 50% of the maximal activity of RARβ2 determined by extrapolation from the plotted points.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed essentially as described previously [Scafonas et al., 1997]. The RXRα and each of the RARβ isoform proteins were recombinant S-Tag fusion proteins prepared in BL21 cells as described under retinoic acid binding assays. BL21 cell extract (25 μg total protein) containing the indicated recombinant proteins were incubated in 25 mM Tris, pH 7.9, 125 mM NaCl, 2.5 mM EDTA, 25 mM dithiothreitol, 12.5 mM $MgCl_2$, 12.5% sucrose, 12.5% glucose, 0.5% Nonidet P-40 (NP-40), and 2.6 μg salmon sperm DNA (Sigma) containing a ^{32}P-labeled RARE probe. The RARE probe was obtained by annealing two complementary single-stranded oligonucleotides (5′-TCGAGGGTAGGGTTCACCGAAAGTTCAC3-′) and (5′-CGAGTGAACTTTCGGTGAACCCTACCCT3-′, which contains the RARE in the RARβ2 promoter (positions −63 to −33 relative to the start site of transcription) [de The et al., 1990]. The resulting double stranded RARE DNA was filled in with Klenow DNA polymerase (Promega) in the presence of [α^{32}P]dCTP (111 Tbq/mmol −3,000 Ci/mmole; Dupont NEN). Unlabeled cold RARE DNA was used as a competitor at a 100-fold excess. The RAR.RXR/DNA complexes were resolved by electrophoresis through a 6% polyacrylamide gel containing 2.5% glycerol in 0.5× TBE (0.09 M Tris borate, pH 8.2, 0.002 M EDTA) at 200 V for 3 h. The gels were dried and exposed to Kodak XRP x-ray film at −70°C.

Retinoic Acid Binding Assays

The K_d value of the RARβ isoforms for all-*trans*-RA and 9-*cis*-RA was determined essentially as described previously using recombinant S-Tag RAR protein [Wolfgang et al., 1997; Scafonas et al., 1997]. Each pET29a-RARβ iso-

TABLE I. Transactivation Activity, Retinoic Acid Binding and Anti-AP1 Activity of RARβ Isoforms

RARβ	EC_{50} (nM)[a]	K_d (nM)[b] all-*trans*-RA	K_d (nM)[b] 9-*cis*-RA	Inhibition (%) of AP1 activity at 10^{-6} M RA[c]
RARβ1	0.4	1.4 ± 0.3	ND[d]	50
RARβ2	0.4	1.4 ± 0.2	2.3 ± 0.2	50
RARβ3	0.4	1.2 ± 0.2	ND[d]	50
RARβ4	10–100	12.1 ± 0.8	11.2 ± 0.5	0

[a]Values taken from Figure 1.
[b]Values taken from Figures 3 and 4. Values are mean ± SE of 4–11 independent measurements.
[c]Values taken from Figure 5.
[d]Not determined.

form prokaryotic expression construct was transformed into BL21(DE3) cells (Novagen). The expression of each RARβ isoform protein and the preparation of the receptor extracts was performed as previously described [Wolfgang et al., 1997; Zhang et al., 1998]. The production of each S-Tag RARβ isoform protein in the receptor extracts was monitored using the S-Tag Western blot kit (Novagen). The Western blot analysis of RARβ1, β2 and β3 demonstrated a major band that migrated at the approximate molecular mass of 53–55 kDa, while RARβ4 migrated at the approximate molecular mass of 48 kDa (data not shown).

Retinoic acid binding assays were performed with receptor extracts diluted with binding buffer (40 mM Hepes, pH 7.9, 120 mM KCl, 10% glycerol, 0.1% (w/v) gelatin, 1 mM EDTA, 4 mM dithiothreitol, and 5 μg/ml each of the protease inhibitors aprotinin and leupeptin) to final concentration of 10–30 μg of total protein. Total RA binding was determined in the diluted protein extracts by adding [^{3}H]all-*trans*-RA (1.82-1.92 Tbq/mmol or 49.2–52.0 Ci/mmol; NEN Life Science Products) or [^{3}H]9-*cis*-RA (1.74 Tbq/mmol or 47.2 Ci/mmol; Amersham) within a concentration range of 0.1–100 nM and incubating for 3 h at 27°C. Dilutions of RA were made in ethanol. In a duplicate set of reactions nonspecific binding was determined in the presence of 200-fold molar excess of unlabeled all-*trans*-RA. The contribution of ethanol to the final volume was the same for each RA concentration and was equal to 2%. Bound RA was separated from free by extraction with 3% (w/v) equal particle size charcoal-dextran prepared as described by Dokoh et al. [1981]. All steps in the procedure were performed under yellow light. Specific RA binding was determined by subtracting the nonspecific binding, always less than 12% of the total binding, from the total binding. No specific RA binding was detected in receptor extracts prepared from cells containing pET29a-RAR expression construct, which were not induced with isopropyl-1-thio-β-D-galactopyranoside or in receptor extracts prepared from cells containing the empty pET29a plasmid treated with isopropyl-1-thio-β-D-galactopyranoside. Apparent equilibrium dissociation constants (K_d) were determined for each RARβ isoform by Scatchard analysis [Scatchard, 1946]. Binding assays for each RARβ isoform were repeated at least four times using two independently prepared receptor extracts.

Anti-AP1 Assays

Anti-AP1 assays were performed essentially as described previously [Soprano et al., 1996]. SK-OV-3 cells were plated at 500,000 cells/60-mm dish. The next day the cells were transfected with the amounts of each DNA indicated in the legends of Figures 5 and 7 by the Ca^{2+} phosphate methodology. The pCAT-AP1 reporter construct contains a triplet AP-1 consensus sequence (5′-GACTCATGACTCATGACTCA3-′) cloned into the *Bgl*II site of p-CAT-promoter (Promega, Madison, WI) [Soprano et al., 1996]. At 24 h after transfection the cells were treated with all-*trans*-RA ranging in concentration from 10^{-11} to 10^{-5} M prepared in ethanol or ethanol carrier. After an additional 24 h, the cells were harvested for CAT [Seed et al., 1988] and β-galactosidase [Eustice et al., 1991] assays. CAT activity was normalized with respect to β-galactosidase activity to control for efficiency of transfection. The % AP1 activity was

calculated using the ethanol carrier sample for each treatment as 100%.

Western Blot

The level of RARβ2 and RARβ4 protein in transfected SK-OV-3 cells was determined by Western Blot Analysis essentially as described previously [Tairis et al., 1994]. Briefly, nuclear protein extracts were prepared 48 h after transfection of SK-OV-3 cells with the DNAs indicated in the legend of Figure 6. Nuclear protein samples were fractionated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 5% stacking gel and a 10% resolving gel. Proteins were electroblotted to polyvinylidene difluoride membranes (Millipore Immobilon-P) according to the method of Burnette [1981]. Immunoblotting with the rabbit RARβ polyclonal antibody (Santa Cruz Biotechnology) and detection using the enhanced chemiluminescence (ECL) kit (Amersham) was performed essentially as described by Ali et al. [1992], with the exceptions noted by Tairis et al. [1994].

RESULTS

Transactivation Activity of RARβ Isoforms

The transactivation activity of each of the four RARβ isoforms was measured in CV-1 cells utilizing a reporter construct, which contains the promoter of RARβ2 including its RARE DNA sequence linked to the CAT gene (RARE-CAT). Figure 1 shows the RA concentration-dependent transactivation activity and Table I lists the EC_{50} values of the four RARβ isoforms. RARβ1, β2, and β3 have very similar RA-dose dependent transactivation activity with an EC_{50} value of approximately 0.4 nM RA.By contrast, RARβ4 has a drastically reduced transactivation activity as compared with that of the other three RARβ isoforms. RARβ4 transactivation activity at 10^{-6} M RA is only approximately 55% that of the other three isoforms and its EC_{50} value is in the range of 10–100 nM RA. It is difficult to determine a more precise EC_{50} value for RARβ4 because this isoform displays little RA-dependent dose-response induction of transactivation activity between the concentrations of 10^{-10} M and 10^{-6} M RA.

Figure 2 demonstrates that the lower transactivation activity of RARβ4 is not due to a reduction in its ability to bind DNA or dimerize with RXR. Electrophoretic mobility shift assays (EMSA) demonstrate that RARβ4 heterodimerizes with RXRα and binds a RARE similar to that of the other three RARβ isoforms.

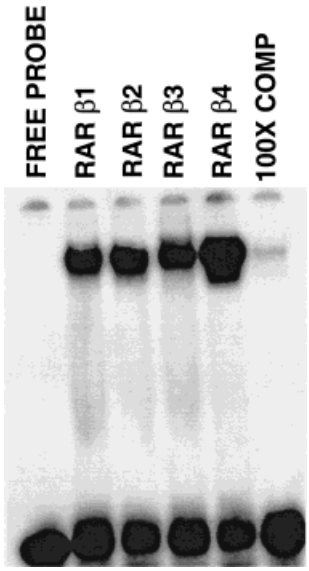

Fig. 2. DNA binding and dimerization of the 4 RARβ isoforms. Bacterial extracts containing recombinant RXRα and the indicated RARβ isoform recombinant proteins (β1, β2, β3, and β4) were incubated with ^{32}P-labeled RARE probe; 100-fold molar excess of unlabeled RARE was added to the RXRα/RARβ2 mixture as a competitor (COMP). After the binding reaction, the mixture was resolved by electrophoresis through a 6% polyacrylamide gel containing 2.5% glycerol, and the retarded bands were visualized by autoradiography.

RA Binding Activity of RARβ Isoforms

We then examined the affinity of each of the RARβ isoforms for all-*trans*-RA and 9-*cis*-RA. The saturation curves and Scatchard plots for all-*trans*-RA and 9-*cis*-RA are shown in Figures 3 and 4, respectively; and the K_d values are listed in Table I. The K_d values of RARβ1, β2 and β3 for all-*trans*-RA are very similar ranging from 1.2 to 1.4 nM, while the K_d value of RARβ4 is elevated approximately 8- to 10-fold to 12.1 nM. Furthermore, the K_d value of RARβ4 for 9-*cis*-RA is elevated approximate 5-fold as compared with that of RARβ2 (11.2 nM compared with 2.3 nM).

Anti-AP1 Activity of RARβ Isoforms

We previously demonstrated that SK-OV-3 cells are resistant to the growth inhibitory action of RA and lack RA-dependent anti-AP1

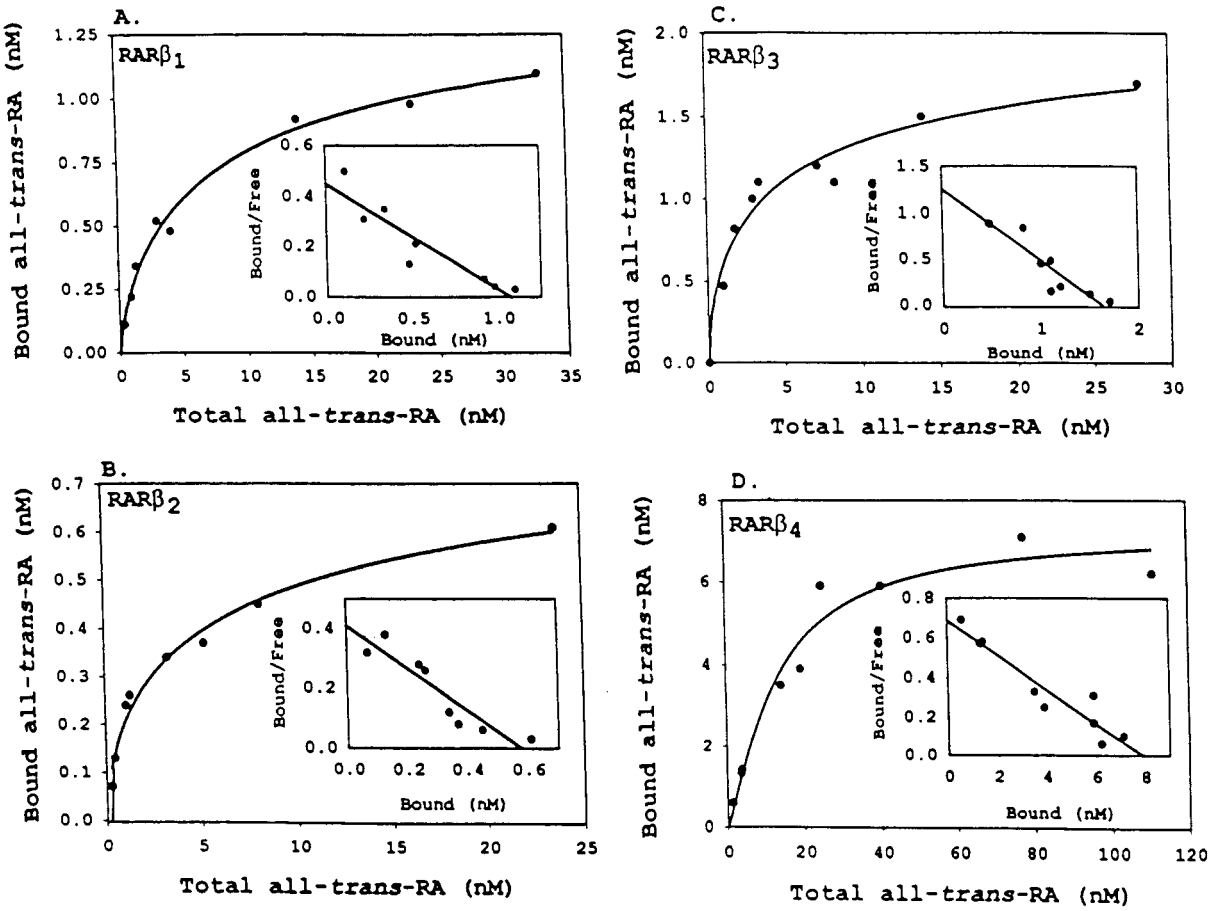

Fig. 3. All-*trans*-RA binding properties of the 4 RARβ isoforms. Specific binding of [^{3}H]-all-*trans*-RA to RARβ1 (**A**), RARβ2 (**B**), RARβ3 (**C**), and RARβ4 (**D**) from a representative experiment is shown. Similar amounts of bacterial extract protein were used in each assay without normalization for the level of expression of the recombinant protein, resulting in differences in the maximum bound all-*trans*-RA between the different RARβ isoform protein preparations. **Insets:** Representative Scatchard plot used to calculate the apparent K_d values of each isoform presented in Table I.

activity. Overexpression of any one of the three RARs along with RXR in SK-OV-3 cells restores RA-dependent growth inhibition and anti-AP1 activity [Soprano et al., 1996]. This makes these cells ideal for the comparison of the anti-AP1 activity of the four RARβ isoforms. Figure 5 and Table I show the RA-dependent anti-AP1 activity of the four RARβ isoforms in SK-OV-3 cells. An RA-dose dependent reduction in AP1 activity is observed in SK-OV-3 cells transfected with RAR β1, β2 and β3. An approximately 50% reduction in AP1 activity was observed in cells treated with 10^{-6} M RA. By contrast, no anti-AP1 activity is observed in cells transfected with RARβ4 at a concentration of RA up to 10^{-6} M, similar to cells transfected with the empty vector pOPRSVI.

Since no anti-AP1 activity was observed in the SK-OV-3 cells transfected with the RARβ4 expression construct DNA and treated with RA, we then determined whether RARβ4 was expressed in these cells. Figure 6 is a Western blot analysis of nuclear protein extracts isolated from SK-OV-3 cells transfected with either RARβ2 or RARβ4 DNA and probed with a RARβ-specific antibody. RARβ4 is expressed in the transfected cells at a level slightly higher than the RARβ2 transfected cells, indicating that the lack of anti-AP1 activity by RARβ4 is not due to poor protein expression.

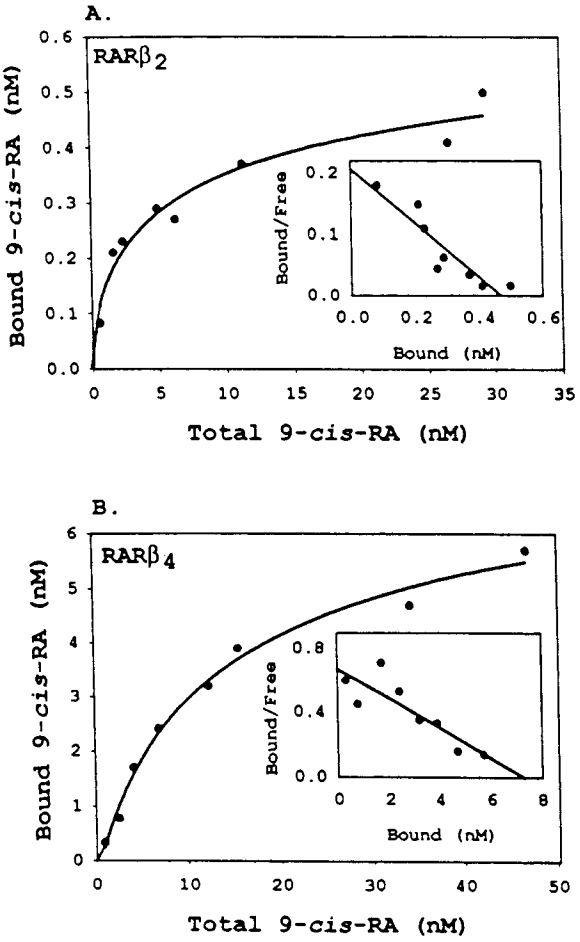

Fig. 4. 9-*cis*-Retinoic acid (RA) binding properties of RARβ2 and RARβ4 isoforms. Specific binding of [^{3}H]-9-*cis*-RA to RARβ2 (**A**) and RARβ4 (**B**) from a representative experiment is shown. Similar amounts of bacterial extract protein were used in each assay without normalization for the level of expression of the recombinant protein, resulting in differences in the maximum bound 9-*cis*-RA between the different RARβ isoform protein preparations. **Insets:** Representative Scatchard plot used to calculate the apparent K_d values for each isoform presented in Table I.

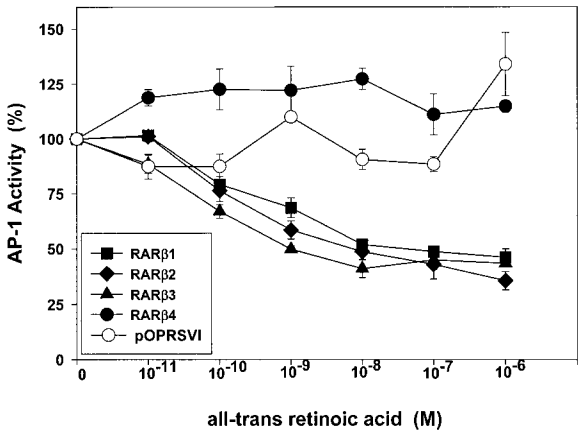

Fig. 5. Retinoic acid (RA)-dependent anti-AP1 activity of the 4 RARβ isoforms. SK-OV-3 cells were transfected with 5 μg of the indicated pOPRSVI RARβ isoform DNA or empty pOPRSVI empty vector DNA, 5 μg of pSG-5 RXRα DNA, 5 μg pCAT-AP1 DNA and 1 μg CMV-βgal DNA. The cells were treated with the indicated concentrations of all-*trans*-RA or ethanol carrier 24 h after transfection. At 48 h after transfection, the cells were harvested and assayed for CAT and βgal activity. 100% AP1 activity is the amount of normalized CAT activity detected in cells treated with ethanol carrier only. Each data point represents the mean of at least four independent experiments performed in duplicate ±S.E. (bars).

Because RARβ4 lacks anti-AP1 activity, we attempted to determine whether RARβ4 could act in a dominant negative fashion on the inhibition of AP1 activity observed with RARβ2. Figure 7 shows the amount of AP1 activity in cells transfected with various amount of RARβ2 and RARβ4 DNA and treated with 10^{-6} M RA . Cells transfected with RARβ2 DNA alone displayed an approximately 50% reduction in AP1 activity. When cells were transfected with RARβ2 DNA and an increasing amount of RARβ4 DNA, the amount of the RARβ2-dependent inhibition of AP1 activity was reduced such that at a DNA ratio of 3:1 (RARβ4:RARβ2), only a 10% reduction in AP1 activity was observed, similar to that observed when the cells were transfected with RARβ4 DNA alone.

DISCUSSSION

One of the many issues concerning the steroid/thyroid hormone superfamily that remains unresolved is why so many of its members exist as multiple receptor isoforms, many of, which vary in the N-terminal A/B domain. In addition to the RAR receptor isoforms, several other members of this superfamily, including the mineralcorticoid, progesterone, and androgen receptors, have been demonstrated to display similar multiple isoforms, which vary at the N-terminal end of the protein [for review, see Keightley, 1998]. The role that each of these isoforms plays in mediating ligand-dependent cellular functions, including that of the RAR isoforms in vitamin A-dependent events, is not well understood. One report has compared the ability of two RARα isoforms (1 and 2) to regulate cell proliferation in newt limb cells. RARα1 was found to be necessary for the regulation of cell proliferation in the

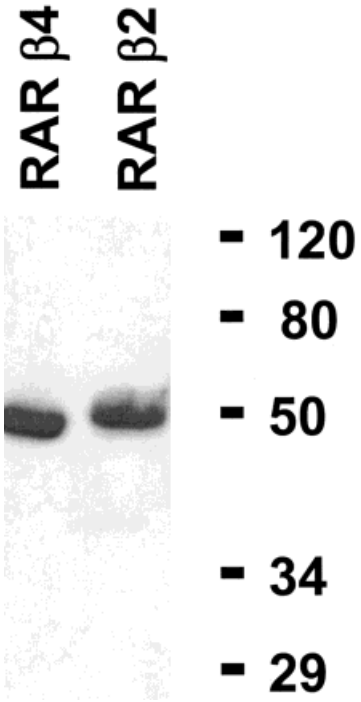

Fig. 6. Expression of RARβ2 and RARβ4 in transfected SK-OV3 cells. SK-OV-3 cells were transfected with 5 μg of either pOPRSVI RARβ2 or pOPRSVI RARβ4 DNA, 5 μg of pSG-5 RXRα DNA, 5 μg pCAT-AP1 DNA and 1 μg CMV-βgal DNA. At 48 h after transfection, nuclear extracts were prepared and 30 μg of total nuclear protein were loaded per lane on a discontinuous SDS-polyacrylamide gel composed of a 5% stacking gel and a 10% resolving gel. Proteins were electroblotted to a polyvinylidene difluoride membrane, and RARβ was detected by immunoblotting with a rabbit anti-RARβ polyclonal antibody followed by a goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence kit. The standard molecular mass markers shown are β-galactosidase (120 kDa), bovine serum albumin (80 kDa), ovalbumin (50 kDa), carbonic anhydrase (34 kDa), and soybean trypsin inhibitor (29 kDa).

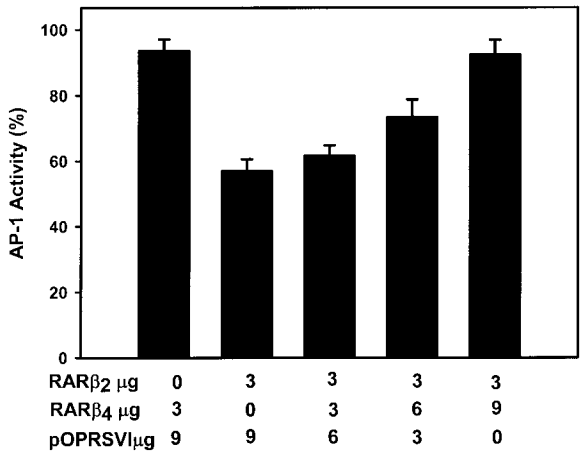

Fig. 7. The dominant negative effect of RARβ4 on RARβ2-dependent anti-AP1 activity. SK-OV-3 cells were transfected with indicated amounts of pOPRSVI RARβ2, pOPRSVI RARβ4 and pOPRSVI empty vector DNA totaling 12 μg, 3 μg of pSG-5 RXRα DNA, 3 μg pCAT-AP1 DNA and 1 μg CMV-βgal DNA. At 24 h after transfection the cells were treated with 10^{-6} M all-*trans*-RA. At 48 h after transfection, the cells were harvested and assayed for CAT and βgal activity. 100% AP1 activity is the amount of normalized CAT activity detected in cells treated with ethanol carrier. Each data point represents the mean of at least four independent experiments performed in duplicate ±S.E. (bars).

developing newt limb buds, while RARα2 did not display this activity [Gann et al., 1996]. Similar studies examining the role of the 4 different RARβ isoforms in mediating RA-dependent cellular events have not been reported.

The purpose of this report was to compare the transactivation activity, ligand binding affinity and anti-AP1 activity of the four murine RARβ isoforms. Currently, the vast majority of information available concerning RARβ is limited to the RARβ2 isoform, and much less is known about the other three isoforms. Our data demonstrate that RARβ1, RARβ2 and RARβ3 have a very similar EC_{50} value in transactivation assays, K_d value for all-*trans*-RA and 9-*cis*-RA and percentage inhibition of AP1 activity. By contrast, RARβ4 displayed a 5- to 10-fold higher K_d for both all-*trans*-RA and 9-*cis*-RA, a greatly elevated EC_{50} value in RA-dependent transactivation assays and no anti-AP1 activity.

It is not surprising to find that the EC_{50} value of RARβ4 is elevated compared with that of the other three RARβ isoforms. The A domain of RARβ4 is only 4 amino acids long and therefore lacks the amino acids associated with AF-1 activity in other RAR isoforms. AF-1 and AF-2 have been demonstrated to act synergistically to enhance RA-dependent transcriptional activity [Nagpal et al., 1992b, 1993]. The inability of AF-1 and AF-2 to synergize due to the absence of AF-1 in RARβ4 could explain the lack of RA-dependent increase in transcriptional activity by this isoform.

By contrast, the finding that the K_d value of RARβ4 for both all-*trans*-RA and 9-*cis*-RA is elevated as compared with that of the other three isoforms is surprising. Since it is generally accepted that the ligand binding properties of RARs lie in the E domain, it is unlikely that any amino acids in the A domain of RARβ

make direct contact with RA. Direct conformation of this must await the solution of the crystal structure of full length RAR or RXR. The crystal structure of only the ligand binding domains of apoRXRα and holoRARγ has been reported [Bourguet et al., 1995; Renaud et al., 1995]. Recently, Shao et al. [1998] demonstrated that ligand binding by PPARγ is modulated by intramolecular communication between the N-terminal A/B domain and the C-terminal E domain of this receptor. Similar to PPARγ it is likely that there is intramolecular communication between the A/B domain and the E domain of other receptors including RARβ, which is responsible for the high affinity binding of ligand. Therefore, it is possible that this reduced affinity of RARβ4 for RA is related to a subtle conformational difference in the three-dimensional structure of the protein caused by the extremely short A domain.

Although the significance of this reduced RA-binding and RA-dependent transactivation activity of RARβ4 is not clear, it is possible that RARβ4 binds with higher affinity another metabolite of RA. By contrast, it is possible that RARβ4 might play an unique role in physiological circumstances where RA levels are elevated. No data have been found that directly compare the levels of RARβ2 and RARβ4 in cells or in tissues treated with increasing amounts of RA. This is particularly interesting since RARβ2 and RARβ4 expression is under the control of the same promoter, which contains a strong RARE [Zelent et al., 1991; de The et al., 1990].

The most intriguing finding is that RARβ4 also lacks anti-AP1 activity and that RARβ4 overexpression can reduce the level of anti-AP1 activity by RARβ2. Although the mechanism underlying the anti-AP1 activity of RARs is not well understood, it has been suggested that inhibition of AP1 activity by RARs is due to the competition between RARs and AP1 for limiting amounts of the coactivator CREB-binding protein (CBP)/p300 in the nucleus [Kamei et al., 1996]. Recently CBP/p300 has been demonstrated to interact directly with both the DEF domain and the A/B domain of PPARγ2 [Gelman et al., 1999]. Interestingly, several other reports have also demonstrated that another coactivator (SRC-1) interacts both with AF-2 in the ligand binding domain and AF-1 in the A/B domain of the androgen receptor [Bevan et al., 1999; Onate et al., 1998; Ikonen et al., 1997]. These data suggest that the stable binding of at least some transcriptional coactivators to nuclear receptors may require interaction of the coactivator with several domains of the receptor including the A/B domain and the DEF domain. It is therefore possible that the lack of anti-AP1 activity by RARβ4 is due to its inability to stably bind and compete with AP1 for a limiting amount of an essential coactivator such as CBP.

Finally, the reduced transactivation activity, ligand binding activity and lack of anti-AP1 activity of RARβ4 is further evidence that suggests that at least one isoform of RARβ has unique functions within a cell. Although in these studies we did not observe any differences between the other three RARβ isoforms (β1, β2 and β3), it is likely given their lack of similarity in the A domain amino acid sequence that these isoforms will display unique functions in other cell systems or in the regulation of other RA-responsive genes.

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