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Biochemistry

All-*trans*-retinol is a ligand for the retinoic acid receptors

(vitamin A/all-*trans*-retinoic acid/ligand binding/neuroblastoma/fusion protein)

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ABSTRACT **Competition of all-*trans*-retinol and all-*trans*-retinaldehyde with ^{3}H-labeled all-*trans*-retinoic acid (RA) for binding to retinoic acid receptors (RARs) was examined in human neuroblastoma cell nuclear extracts. All-*trans*-retinol was 35-fold less potent than all-*trans*-RA, whereas all-*trans*-retinaldehyde was 500-fold less active in binding to the nuclear receptors. To confirm that all-*trans*-retinol binds to RARs, experiments were carried out with RARs α, β, and γ expressed as bacterial fusion proteins. All-*trans*-retinol was only 4- to 7-fold less potent than all-*trans*-RA in binding to all three RAR subtypes. The all-*trans*-retinol binding observed was not the result of metabolism of retinol to RA or some other active compound during the binding experiment. Retinyl acetate was virtually inactive in competition binding experiments, while very slight activity was observed with 13-*cis*-RA and all-*trans*-retinaldehyde. Significant competition occurred with 4-hydroxy-RA and 4-keto-RA, which were 15- to 40-fold less potent than all-*trans*-RA. The 9-*cis* isomer of RA was equipotent with all-*trans*-retinol in these studies. These results suggest that all-*trans*-retinol cannot be excluded as a physiologically significant ligand for RAR-mediated gene expression.**

Vitamin A (retinol) and its metabolites play important roles in vision, growth, and reproduction. Retinol, by virtue of its conversion to retinaldehyde, supports the visual requirement for vitamin A. Retinaldehyde is further converted to retinoic acid (RA), which is believed to represent the form of the vitamin that is active in cellular growth and differentiation. RA exerts its effects, at least in part, by binding to nuclear receptor proteins of the RA receptor (RAR) and retinoid X receptor (RXR) families (1, 2). The binding of RA to its receptor is followed by the activation or inhibition of transcription of retinoid-responsive genes. All-*trans*-RA is a high-affinity ligand for all three RAR subtypes: α, β, and γ (3–9). 9-*cis* RA is the highest-affinity ligand for the RXRs identified to date (3, 10, 11). In addition to the nuclear receptors, cellular RA-binding proteins (CRABPs) and cellular retinol-binding proteins (CRBPs) are present in many cells (12). They may play important roles in regulating retinoid metabolism and the amount of compound that gains access to the nuclear receptors (13–16).

RAR mRNAs and proteins have been identified and characterized in a number of RA-responsive cells and tissues, including human neuroblastoma cells (17). These cells express mRNAs for RARs α and γ before exposure to RA, and express a 2.9-kb RARβ mRNA within hours of exposure to RA. Nuclear extracts prepared from human neuroblastoma cells exhibit saturable, high-affinity binding of all-*trans*-[^{3}H]RA. In this report we show that all-*trans*-retinol is effective in competing with all-*trans*-[^{3}H]RA for binding to the nuclear receptors. Further, we demonstrate that all-*trans*-retinol binds to RARs α, β, and γ expressed as fusion proteins in *Escherichia coli*.

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MATERIALS AND METHODS

Chemicals. All-*trans*-[^{3}H]RA (50.6 Ci/mmol) and all-*trans*-[^{3}H]retinol (37.1 Ci/mmol; 1 Ci = 37 GBq) were obtained from New England Nuclear and were checked for radiochemical purity (>95%) by HPLC (18). All-*trans*-retinol, all-*trans*-RA, 13-*cis*-RA, all-*trans*-retinaldehyde, and all-*trans*-retinyl acetate were purchased from Eastman Kodak. 9-*cis*-RA was a generous gift from H. F. DeLuca (Department of Biochemistry, University of Wisconsin–Madison). The retinoids 4-hydroxy-RA and 4-keto-RA were kindly provided by Hoffman–La Roche. Stock solutions of retinoids were prepared in ethanol and stored under argon at −70°C. Concentrations of retinoid-containing solutions were determined by spectrophotometry, and purities (>95%) were confirmed by reverse-phase HPLC (18). All manipulations involving retinoids were performed under amber light.

Cell Culture. The clonal human neuroblastoma cell line LA1-15n (19) was a gift from J. Biedler (Memorial Sloan–Kettering Cancer Center, New York). Cells were maintained as described (17).

Construction of Expression Plasmids. The pATH expression vectors were used for the production of RAR fusion proteins in *E. coli* (20) and were a generous gift from A. Tzagoloff (Columbia University, New York). Plasmids containing full-length cDNAs for the human RARs α, β, and γ were gifts from R. Evans (Howard Hughes Medical Institute, La Jolla, CA), M. Pfahl (La Jolla Cancer Research Foundation, La Jolla, CA), and P. Chambon (Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Institut de Chimie Biologique, Strasbourg, France), respectively. The multiple cloning sites of pATH20 and pATH1 expression plasmids were modified by the addition of synthetic oligonucleotides between the *Sac* I and *Hin*dIII sites (5′-gagctCAGGTACCAAGCATGCAGATCTAagctt-3′) and between the *Bam*HI and *Hin*dIII sites (5′-gGATCCCCATGGCCACACTGCAGAGATCTAagctt-3′), respectively, to facilitate subcloning of human RAR fragments. A 2.7-kb fragment of the human RARα cDNA was excised from the pHK$_1$ vector (7) with *Kpn* I and *Bgl* II and ligated into the modified pATH20. This resulted in the addition of RARα nt 155–2876 and the construct is referred to as RARα/pATH. A 1.5-kb fragment of the human RARβ cDNA was excised from the B1-RARβ plasmid (4) with *Sph* I and *Hin*dIII and ligated into the modified pATH20, resulting in the addition of nt 424–1913 (RARβ/pATH). A 1.5-kb fragment of the human RARγ cDNA was excised from RAR$_{\gamma D}$/pSG5 (9) vector with *Nco* I and *Bgl* II and inserted into the modified pATH1 vector, resulting in the addition of nt 48–1538 (RARγ/pATH). *E. coli* cells were transformed with the resulting plasmids (21).

Production of RAR/pATH Fusion Proteins and Receptor-Containing Extracts. Expression of fusion proteins was per-

Abbreviations: RA, retinoic acid; RAR, retinoic acid receptor; CRBP, cellular retinol-binding protein; RAR/pATH, anthranilate synthase–RAR fusion; RAR/BCV, baculovirally expressed RAR.
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formed as described (20) with minor modifications. All buffers used in cell lysis and in all subsequent manipulations included phenylmethylsulfonyl fluoride (1 mM) and soybean trypsin inhibitor (10 μg/ml). Presence of the RAR/pATH fusion proteins was confirmed by resolution of samples in SDS/polyacrylamide gels, followed by staining with Coomassie blue dye.

The RAR/pATH fusion proteins in *E. coli* extracts were present largely as insoluble inclusion bodies. Therefore, the expressed proteins were renatured before use in binding experiments (22). Whole-cell extracts containing baculovirally expressed murine RARγ (RARγ/BCV) were prepared as described (23). The murine RARγ/BCV was a gift from H. F. DeLuca. Nuclear extracts were prepared from human neuroblastoma cells (17).

All-*trans*-[^{3}H]RA Binding Studies. For equilibrium saturation binding analyses, renatured RAR fusion proteins (0.001–0.017 mg of total protein) or receptor-containing cell nuclear extracts (≈0.3 mg of total protein) were incubated on ice until equilibrium was reached (3 hr) with variable concentrations of all-*trans*-[^{3}H]RA in the absence or presence of a 100-fold excess of unlabeled all-*trans*-RA to assess nonspecific binding. For competition binding studies, receptor-containing samples were incubated with a constant amount of all-*trans*-[^{3}H]RA in the presence of various concentrations of competing unlabeled retinoid or the equivalent volume of ethanol (4% final concentration). Receptor–ligand complexes were separated from free ligand by use of hydroxylapatite (24, 25). The quantity of all-*trans*-[^{3}H]RA remaining in the hydroxylapatite pellet was analyzed by liquid scintillation counting. Binding constants were calculated with the LIGAND computer program (26). Sucrose density sedimentation studies were performed as described (17).

Identification of the Retinoid Bound to Bacterially Expressed RARγ. Following saturation equilibrium binding of ^{3}H-labeled ligands to RARγ/pATH, radiolabeled retinoid was extracted from receptor bound to hydroxylapatite and subjected to HPLC analysis in order to confirm the identity of the bound tritiated ligand. Unlabeled retinoids (10 μM all-*trans*-RA and 5 μM all-*trans*-retinol) were included in the extraction solvent to protect the labeled ligand from oxidation. They also served as internal standards. The organic extracts were dried under argon, resuspended in HPLC solvent, and analyzed by reverse-phase HPLC (Zorbax ODS column of 4.6 mm × 25 cm; flow rate, 1 ml/min) using acetonitrile/10 mM sodium acetate (80:20). Elution profiles were monitored at 340 nm and fractions were collected at 12-sec intervals for liquid scintillation counting.

RESULTS

Competition of All-*trans*-Retinol for Binding of All-*trans*-[^{3}H]RA to RARs in Nuclear Extracts from Human Neuroblastoma Cells. In sedimentation and competition binding analyses, all-*trans*-retinol effectively competed with all-*trans*-[^{3}H]RA for binding to nuclear extract proteins prepared from human neuroblastoma cells (Fig. 1). Fractionation of all-*trans*-[^{3}H]RA-labeled nuclear extracts on 4–20% sucrose density gradients revealed a single peak (≈4 S) of specific all-*trans*-[^{3}H]RA-binding activity. Unlabeled all-*trans*-RA (500 nM) completely eliminated binding of all-*trans*-[^{3}H]RA to the 4S species, whereas the inclusion of all-*trans*-retinol (500 nM) resulted in a 53% reduction in specific binding. All-*trans*-retinaldehyde was much less effective than RA and retinol in competing with all-*trans*-[^{3}H]RA for binding to the nuclear receptor. All-*trans*-retinol was ≈35-fold less potent than all-*trans*-RA in competing with all-*trans*-[^{3}H]RA for binding to RAR in crude neuroblastoma nuclear extracts (K_i = 0.09 nM for RA and 3.1 nM for retinol), whereas all-*trans*-retinaldehyde was ≈500-fold less effective than all-*trans*-RA

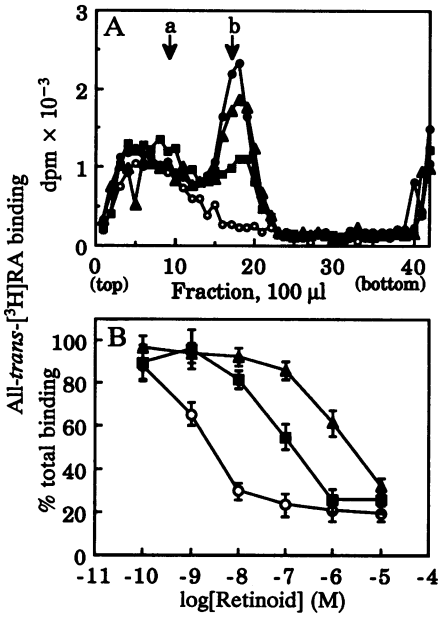

FIG. 1. Retinoid binding in nuclear extracts prepared from human neuroblastoma cells. (*A*) Sedimentation analysis of specific all-*trans*-[^{3}H]RA binding. Nuclear extract was incubated with 5 nM all-*trans*-[^{3}H]RA in the absence (●) or presence of a 100-fold excess of unlabeled retinoid: all-*trans*-RA (○), all-*trans*-retinol (■), or all-*trans*-retinaldehyde (▲). Samples were treated with dextran-coated charcoal and analyzed on 4–20% sucrose gradients. Arrows show the migration of ^{14}C-labeled proteins used as internal sedimentation standards: cytochrome *c* (a, 1.8 S) and ovalbumin (b, 3.7 S). (*B*) Equilibrium competition binding studies. Nuclear extract was incubated with 1 nM all-*trans*-[^{3}H]RA in the absence (defined as 100% total binding) or presence of various concentrations of unlabeled retinoids (symbols as in *A*). Values shown in *B* are means ± SE.

(Fig. 1*B*). Additional studies in which the RAR was partially purified by chromatography on DNA-cellulose showed that retinol was ≈10-fold less potent than RA in competing for all-*trans*-[^{3}H]RA binding (data not shown).

Expression and Characterization of RAR/pATH Fusion Proteins. To examine retinol binding to RARs more directly, individual receptor subtypes (α, β, and γ) were expressed as fusion proteins in *E. coli* (Fig. 2*A*). Equilibrium saturation binding studies of the renatured RAR/pATH proteins (α, β, and γ) revealed that the binding of all-*trans*-[^{3}H]RA was saturable and that for each receptor subtype, a single class of high-affinity all-*trans*-RA binding sites was present (Fig. 2*B*). No specific binding of all-*trans*-[^{3}H]RA was observed for extracts of *E. coli* containing only pATH20. The equilibrium dissociation constants (K_d; see Fig. 2 legend) obtained for RAR/pATH fusion proteins are in the range of values reported for RARs expressed in other prokaryotic (6, 27, 28) and eukaryotic expression systems (3, 23, 29, 30). The K_d values derived by Scatchard analysis did not differ between full-length RARγ expressed as a fusion protein in *E. coli* and the full-length murine receptor expressed in insect cells (23), thus demonstrating that the presence of N-terminal anthranilate synthase does not reduce the binding affinity of the RAR/pATH proteins for all-*trans*-[^{3}H]RA. Thus, the evidence supports the use of RAR/pATH fusion proteins to assess the relative binding affinities of compounds for the RAR family of receptors.

Competition Binding Studies of RARs Expressed in Bacteria and Insect Cells. Of the compounds tested, all-*trans*-RA showed the highest affinity for the three receptor subtypes expressed as fusion proteins in bacteria (Fig. 3; Table 1). All-*trans*-retinol was quite effective in competing with all-*trans*-[^{3}H]RA for binding to the bacterially expressed recep-

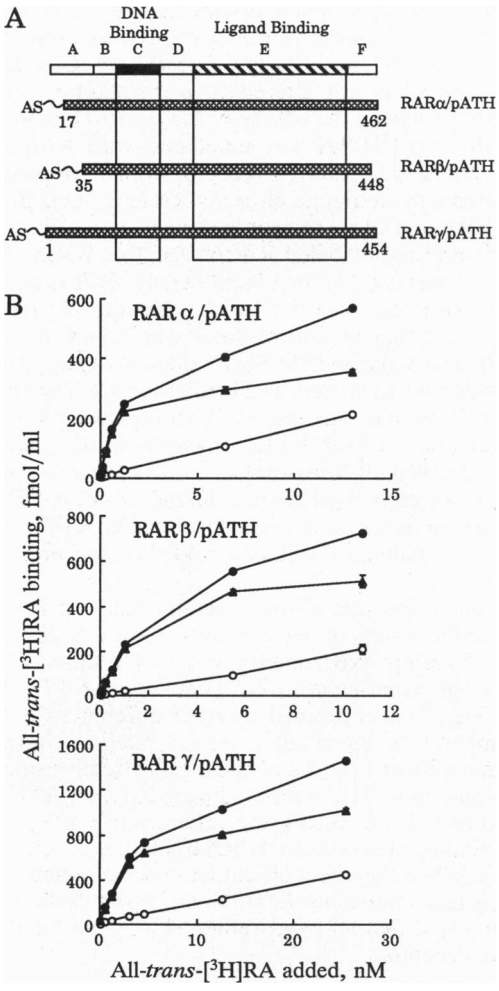

FIG. 2. Expression of human RARα, -β, and -γ as fusion proteins in *E. coli*. (*A*) Schematic depiction of the functional domains of expressed RAR/pATH proteins. Amino acid number is shown below each construction. The 37-kDa portion of anthranilate synthase (AS) at the N terminus is not drawn to scale. (*B*) Equilibrium saturation binding of all-*trans*-[^{3}H]RA by renatured RAR/pATH fusion proteins. Proteins were incubated with various concentrations of all-*trans*-[^{3}H]RA in the presence (○) or absence (●) of unlabeled all-*trans*-RA. Specific binding values (▲) were derived by subtracting nonspecific binding from total binding. Values are means ± SE. Transformation of the data by Scatchard analysis yielded the following K_d values: RARα/pATH, 0.4 nM; RARβ/pATH, 0.8 nM; RARγ/pATH, 3.3 nM.

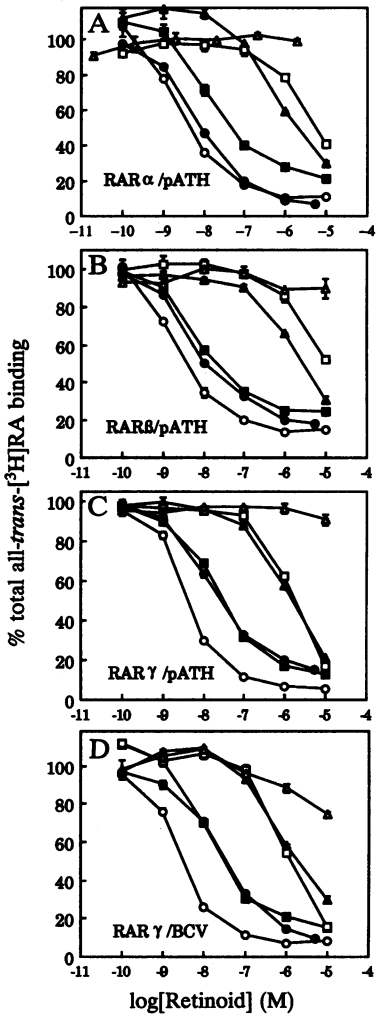

FIG. 3. Competition of retinoids for all-*trans*-[^{3}H]RA binding to RARα/pATH (*A*), RARβ/pATH (*B*), RARγ/pATH (*C*), or RARγ/BCV (*D*). Extracts were incubated with 2 nM all-*trans*-[^{3}H]RA in the absence or presence of various concentrations of unlabeled all-*trans*-RA (○), 9-*cis*-RA (●), all-*trans*-retinol (■), all-*trans*-retinaldehyde (▲), 13-*cis*-RA (□), or all-*trans*-retinyl acetate (△). Values are means ± SE.

tors, and was only 4- to 7-fold less potent than authentic all-*trans*-RA. All-*trans*-retinol and 9-*cis*-RA were nearly equipotent in binding to the three subtypes of receptors. The potency of 9-*cis*-RA in binding to RARs confirms the report of Allenby and coworkers (3). Two RA metabolites, 4-hydroxy-RA and 4-keto-RA, were reasonably effective in competing with all-*trans*-[^{3}H]RA for binding to all three subtypes of RAR (15- to 40-fold less potent than all-*trans*-RA). Less activity was observed with all-*trans*-retinaldehyde and 13-*cis*-RA, whereas retinyl acetate was virtually inactive. These results suggest that the biologic activity of 13-*cis*-RA may result from isomerization to the all-*trans* form *in vivo*. All the retinoids tested showed a similar rank order for potency of competition with all-*trans*-[^{3}H]RA for binding to all three subtypes of bacterially expressed RAR (Fig. 3 *A–C*). There were no differences in the competition binding profiles obtained for RARγ expressed as a fusion protein in bacteria or in the eukaryotic insect cell expression system (Fig. 3 *C* and *D*), and all-*trans*-retinol was able to compete for all-*trans*-[^{3}H]RA binding to all receptor types examined.

The rank order of potency of all-*trans*-RA, all-*trans*-retinol, and all-*trans*-retinaldehyde in competing for all-*trans*-[^{3}H]RA binding to the bacterially expressed RARs parallels the results seen in human neuroblastoma cell nuclear extracts and supports the conclusion that all-*trans*-retinol competes with all-*trans*-[^{3}H]RA for binding to the nuclear RARs. However, all-*trans*-retinol was slightly less potent in competing with all-*trans*-[^{3}H]RA for binding to the

Table 1. Competition for all-*trans*-[^{3}H]RA binding to RAR/pATH fusion proteins

Retinoid	K_i, nM α	β	γ
All-*trans*-RA	0.2	0.3	2.3
All-*trans*-retinol	1.4	1.1	13
All-*trans*-retinaldehyde	≈69	≈130	≈500
9-*cis*-RA	1.0	1.1	18
13-*cis*-RA	≈210	≈280	≈8000
4-Hydroxy-RA	6.7	9.7	35
4-Keto-RA	2.5	7.9	29

nuclear proteins in human neuroblastoma cells than it was in competing for binding to receptors expressed in bacteria and insect cells. This difference was more pronounced in the crude nuclear extract than in a partially purified preparation. Because retinol is a more hydrophobic ligand than RA, nonspecific retinol binding becomes a problem as receptor purity decreases. This is evidenced by an 8- to 10-fold increase in K_i for all-*trans*-retinol that was observed in insect cell-derived RARγ after it was mixed with crude neuroblastoma nuclear extracts (with recombinant receptor contributing >90% of the specific binding activity; data not shown). Small differences in retinol potency could also have resulted from contamination of nuclear extract with other retinoid-binding proteins, such as CRBP, that would not be present in bacterial preparations. Finally, the binding characteristics of RAR isoforms that are present in human neuroblastoma cells may not parallel those of the RARs expressed in *E. coli* or insect cells.

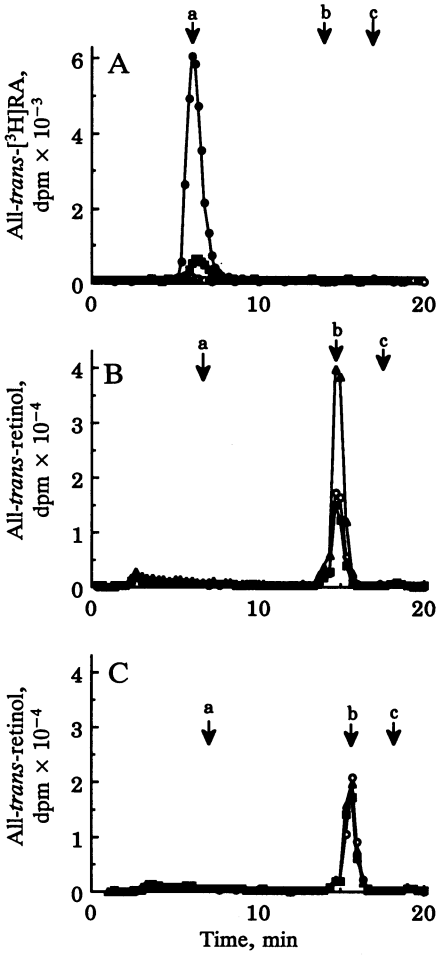

FIG. 4. Reverse-phase HPLC analysis of ^{3}H-labeled retinoid from the modified ligand-trap assay. RARγ/pATH-containing extracts were incubated with 5 nM all-*trans*-[^{3}H]RA (●) (*A*) or 25 nM all-*trans*-[^{3}H]retinol (▲) (*B*) alone or in the presence of a 100-fold excess of unlabeled all-*trans*-RA (○) or all-*trans*-retinol (■). (*C*) Extract prepared from cells containing the pATH parent vector was incubated with all-*trans*-[^{3}H]retinol as in *B*. The elution position of authentic standards that were included as internal controls is shown: a, all-*trans*-RA; b, all-*trans*-retinol; c, all-*trans*-retinaldehyde. A mixture of acetonitrile and butanol (1:1) was effective in extracting ≈80% of the specifically bound all-*trans*-[^{3}H]RA from the hydroxylapatite pellet. A mixture of chloroform, methanol, and water (4:2:1) followed by two extractions with chloroform and water (1:1) resulted in the extraction of >75% of the all-*trans*-[^{3}H]retinol from samples. Recovery of tritiated retinoid after HPLC ranged from 70% to 100%.

Identification of Receptor-Bound Ligand. A modified receptor-dependent ligand-trap assay (11) was used to prove that all-*trans*-[^{3}H]retinol binds directly to RAR and that all-*trans*-retinol is not altered to a metabolite capable of binding RAR during the course of the *in vitro* binding assay. When all-*trans*-[^{3}H]RA was incubated with RARγ/pATH protein, the major peak of receptor-bound tritiated ligand comigrated with authentic all-*trans*-RA (Fig. 4*A*). Unlabeled all-*trans*-RA and all-*trans*-retinol were effective in competing for binding of radiolabeled RA to receptor. When all-*trans*-[^{3}H]retinol was used in the binding assay, HPLC analysis of retinoid extracted from the hydroxylapatite pellets clearly demonstrated that all-*trans*-retinol was bound to receptor (Fig. 4*B*). The majority (>90%) of radioactivity applied to the HPLC column was present as all-*trans*-retinol. The binding of all-*trans*-[^{3}H]retinol was specific, as this peak of activity was largely eliminated from HPLC fractions in the presence of excess unlabeled all-*trans*-retinol or all-*trans*-RA. An equivalent amount of nonspecifically bound all-*trans*-[^{3}H]retinol was observed in bacterial extract lacking RAR (Fig. 4*C*) and in extract containing the RARγ/pATH fusion protein (Fig. 4*B*).

The conclusion that all-*trans*-retinol binds to RARs conflicts with the results of two previous studies (6, 28). In those studies, binding experiments were conducted at 22°C, whereas our experiments were conducted at 4°C. For this reason, we next determined whether differences in incubation temperature could alter retinol binding. Indeed, we found that all-*trans*-retinol is much less effective in competing with all-*trans*-[^{3}H]RA for binding to RARα/pATH at 22°C than it is at 4°C (Fig. 5). Further, there was a 30% reduction in total binding of all-*trans*-[^{3}H]RA binding at 22°C compared with 4°C. When the effect of temperature on receptor–ligand binding is taken into consideration, the available data support the conclusion that all-*trans*-retinol is a ligand for the RAR family of receptors.

DISCUSSION

The activity of retinol in promoting the growth and differentiation of cells has been proposed to result from its conversion to smaller quantities of other biologically active compounds, particularly all-*trans*-RA (31). All-*trans*-RA, in turn, is believed to exert its effects by binding to the RAR family of receptors (1, 2). Analysis of receptor-containing extracts has shown that all-*trans*-RA is a high-affinity ligand for the RAR family of receptors (3, 5, 6, 27–29). In this report we show that all-*trans*-retinol competes with all-*trans*-[^{3}H]RA for binding to the nuclear receptors in human neuroblastoma

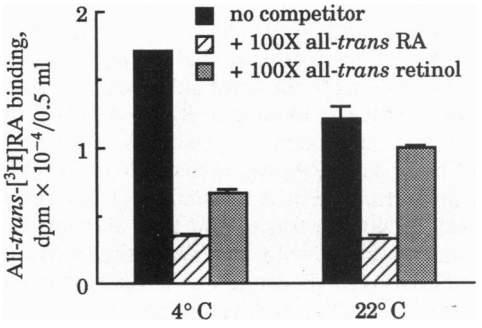

FIG. 5. Effect of incubation temperature on competition of all-*trans*-RA or all-*trans*-retinol for all-*trans*-[^{3}H]RA binding to RARα/pATH. Extract was incubated with all-*trans*-[^{3}H]RA (5 nM) in the absence or presence of a 100-fold excess of unlabeled compound for 3 hr at the temperatures indicated. The hydroxylapatite assay was conducted at 4°C (see *Materials and Methods*.) Values represent means ± SE.

cells, as well as to individual RAR subtypes expressed in bacteria and insect cells. Furthermore, we show that all-*trans*-[^{3}H]retinol can bind directly to RARs.

Several lines of evidence support the conclusion that all-*trans*-retinol binds to the RAR family of receptors. First, sedimentation studies showed that all-*trans*-retinol competed with all-*trans*-[^{3}H]RA for binding to an ≈4S species in human neuroblastoma cell nuclear extracts. This is the sedimentation position expected for a nuclear RAR. Because all-*trans*-RA does not bind to the RXRs (3, 10, 11), all-*trans*-retinol was most likely competing for all-*trans*-[^{3}H]RA binding to the RARs. This is supported by evidence showing that all-*trans*-retinol effectively competed with all-*trans*-[^{3}H]RA for binding to human RARs α, β, and γ expressed as fusion proteins in *E. coli*. Retinol is only 4- to 7-fold less potent in binding to RARs than is all-*trans*-RA. Further, it is equipotent with 9-*cis*-RA, which is a ligand for both the RAR and RXR families (3). The final piece of evidence in support of all-*trans*-retinol binding to RARs comes from the receptor-dependent ligand-trap experiment. Tritiated retinoid specifically bound to RAR was extracted from hydroxylapatite and positively identified as all-*trans*-[^{3}H]retinol by comigration with authentic unlabeled retinol in HPLC analysis. This experiment proves that all-*trans*-retinol is not metabolized or degraded during the course of the incubation to a compound that is active in binding to receptor. Further, it confirms that authentic all-*trans*-retinol is physically associated with RAR.

It may be somewhat difficult to reconcile retinol binding to RARs in light of the abundance of this retinoid in normal physiology. Retinol is the primary circulating form of vitamin A. If retinol can bind to RARs *in vivo*, there are several ways to rationalize why the nuclear receptors are not continuously activated by this abundant retinoid. It is possible that all-*trans*-retinol binds to but does not activate the receptor. Alternatively, it is possible that animals requiring vitamin A have developed a finely tuned system to regulate the amount of endogenous retinoid that is available to cells and ultimately to activate the nuclear receptors. CRBPs in the cytoplasmic compartment of cells may limit the access of free retinol to the nuclear compartment (12, 16). However, in vitamin A excess, retinol may exceed the binding capacity of the CRBPs and directly activate the RARs. The teratogenic potential of all-*trans*-retinol as well as the RA metabolites 4-hydroxy-RA and 4-keto-RA could be explained by their ability to activate the nuclear receptors (32, 33). The idea that all-*trans*-retinol can activate the RARs *in vivo* cannot be ruled out at this time, because all-*trans*-retinol can certainly bind to this family of receptors.

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