Transactivation Properties of Retinoic Acid and Retinoid X Receptors in Mammalian Cells and Yeast

CORRELATION WITH HORMONE BINDING AND EFFECTS OF METABOLISM*

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Elizabeth A. Allegretto‡, Michael R. McClurg §, Steven B. Lazarchik †, David L. Clemmi, Sandra A. Kerner, Marc G. Elgort†, Marcus F. Boehml, Steven K. White**, J. Wesley Pike‡, and Richard A. Heyman§

From Ligand Pharmaceuticals, Inc., Departments of ‡Biochemistry, §Cell Biology, Molecular Biology, and **Chemistry, San Diego, California 92121

The binding affinities of 9-cis-retinoic acid (9-cis-RA) and all-trans-retinoic acid (t-RA) for retinoic acid receptors (RAR) α, β, and γ and for retinoid X receptors (RXR) α, β, and γ were determined using the recombinant receptor proteins and were compared with each hormone's ability to activate transcriptions through the receptors in mammalian and yeast cell systems. 9-cis-RA bound to both the RXRs (Kd = 1.4–2.4 nM) and the RARS (Kd = 0.2–0.8 nM). The ability of 9-cis-RA to bind to the RARS and RXRS correlated with its ability to produce similar transactivation profiles with these receptors in mammalian and yeast cell assays. t-RA bound to the RARS (Kd = 0.2–0.4 nM) and activated transcription through the RARS in mammalian and yeast cells. In contrast, while t-RA did not bind to the RXRS, it did activate the RXRS, albeit less potently than 9-cis-RA, in mammalian cells. In yeast, however, the RXRS activated transcription only in the presence of 9-cis-RA, not with t-RA. While RARγ is activated in yeast by either t-RA or 9-cis-RA, the overall level of transcription was increased upon the addition of hormone-occupied RXR. Metabolism studies suggest that while there was no cell-dependent interconversion between t-RA and 9-cis-RA in yeast, there was cell-dependent conversion of 9-cis-RA to t-RA in mammalian cells.

The retinoids, derivatives of vitamin A, are modulators of cellular proliferation and differentiation as well as effectors of morphogenic changes (Thaller and Eichele, 1987; Roberts and Sporn, 1984; Durston et al., 1989). These actions are thought to be exerted through the nuclear retinoid receptors (for reviews, see Leid et al. (1992a) and Mangelsdorf et al. (1994)) which are members of the steroid receptor superfamily (Evans, 1988; Fuller, 1991). The retinoid receptors are comprised of two superfamilies, the retinoid acid receptors (RARs) and the retinoid X receptors (RXRs). The RARs are encoded by three different genes, α, β, and γ, each subtype expressing several isoforms that differ in their amino termini due to alternative mRNA splicing and different promoters (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Zelent et al., 1988; Krust et al., 1989; Ishikawa et al., 1990). The RXRs are also encoded by three genes, α, β, and γ, and, at present, isoforms of these subtypes have not been described (Mangelsdorf et al., 1990; Leid et al., 1992b; Mangelsdorf et al., 1992). The RARs and RXRs are classified into these two subfamilies on the basis of their (a) primary structural differences, (b) binding affinity characteristics to synthetic and naturally occurring retinoids, and (c) differential regulation of target genes (Mangelsdorf et al., 1994). The complexity of retinoid receptor regulation of gene expression is further complicated by recent findings that RXRs can form heterodimers with the RARs, vitamin D receptor (VDR), and thyroid hormone receptor (TR) subtypes (Yu et al., 1991; Kliwer et al., 1992a; Zhang et al., 1992a), as well as various orphan receptors (Kliwer et al., 1992b, 1992c).

Naturally occurring and synthetic ligands have been described that have distinctive binding properties and transactivation effects on the various RAR and RXR subtypes, thereby allowing differential modulation of retinoid receptor-induced gene expression. For example, t-RA binds directly to the RARs with high affinity, thereby activating the RARs to modulate gene expression. However, t-RA does not bind to the RXRs, and only at high concentrations does it transactivate via the RXRs (Mangelsdorf et al., 1990). This latter observation led to the hypothesis that t-RA was metabolized in cells to a retinoid compound capable of binding to, and thereby activating, the RXRs (Mangelsdorf et al., 1990). Consistent with this hypothesis, we (Heyman et al., 1992) as well as others (Levin et al., 1992b, 1992c) recently reported the discovery of an isomer of t-RA, 9-cis-retinoic acid (9-cis-RA) that binds recombinant human RARα and stimulates transcription of RXR-dependent promoters in cell-based assays. The discovery that 9-cis-RA binds directly to RXRα has led us to further investigate its functional properties and to determine its ability to bind and transactivate through the other described RXRs, RXRβ and RXRγ. Moreover, since 9-cis-RA is an activator of RARα (Heyman et al., 1992), we also examined its ability to bind and modulate the transcriptional properties of the RAR subtypes. Finally, we have compared the receptor-retinoid binding affinities with the ability of 9-cis-RA and t-RA to regulate gene expression in both mammalian cells and yeast. The results demonstrate that repeat with spacing of 1 base between repeats; DR3, HRE containing a direct repeat with spacing of 3 bases; DR5, HRE containing a direct repeat with spacing of 5 bases; PRE, retinoid response element derived from the RARβ promoter; HAP, hydroxylapatite.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Ligand Pharmaceuticals, 11149 N. Torrey Pines Rd., Suite 110, La Jolla, CA 92037. Tel.: 619-550-7817; Fax: 619-625-7010.

§ The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; 9-cis-RA, 9-cis-retinoic acid; t-RA, all-trans-retinoic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonium)-1-propanesulfonate; Emax, concentration eliciting 50% of full response; CRBP, cellular retinol binding protein; HPLC, high performance liquid chromatography; VDR, vitamin D receptor; TR, thyroid hormone receptor; TBS, Tris-buffered saline; RBP, retinol binding protein; PAGE, polyacrylamide gel electrophoresis; HRE, hormone response element; DR1, HRE encompassing a direct
9-cis-RA is a high affinity ligand for members of both the RAR and RXR subfamilies, whereas t-RA only binds the RARs. The transcriptional assays in yeast demonstrate that there is a direct correlation between 9-cis-RA binding to RAR and transcriptional activation by 9-cis-RA through the RXRs, implying that the binding of 9-cis-RA results in an RAR homodimer complex that is transcriptionally active on retinoid responsive promoters. Both 9-cis-RA and t-RA stimulate transactivation in yeast through RARY alone or via an RAR/RXRXR combination, with the highest level of transcription resulting from the heterodimeric complex when both receptors are occupied with ligand.

EXPERIMENTAL PROCEDURES

**Hormones**—All trans-[11,12-3H]retinoic acid (50 Ci/mmol) was obtained from DuPont NEN. Unlabeled all-trans-retinoic acid was from Sigma. 9-cis-[11,12-3H]retinoic acid (29 Ci/mmol) and 9-cis-[11,12,13,14-3H]retinoic acid (50 Ci/mmol) and unlabeled 9-cis-retinoic acid were synthesized and purified at Ligand Pharmaceuticals.

**Antibodies**—Rabbit antiserum against hRARα peptide was kindly provided by J. Dyck (Heyman et al., 1992). We prepared subtype-specific rabbit antisera against hRXRα, mRXRα, hRARβ, mRXRβ, hRARγ and hRARδ, as well as a mouse monoclonal antibody against an hRXRα peptide. A mouse monoclonal antibody against mouse RARβ peptide 423-448 was kindly provided by W. Vedeckis (All et al., 1992). CV-1 Co-transactivation Assays—Assays were performed exactly as described in Heyman et al. (1992). Reporter-luciferase constructs used were the palindromic thyroid hormone response element (TRE-pal) sequence for RAR co-transfections and the CRBPII response element for RXR co-transfections.

**Vector and Plasmid Constructions**—The baculovirus Sf21 insect cell system was used to express each of the six retinoid receptors. Recombinant transfer vectors containing receptor cDNA encoding hRARα (Giguere et al., 1987), hRARβ (Benbrook et al., 1988), hRARγ (Ishikawa et al., 1990), hRXRα (Heyman et al., 1992), mRXRα (Mangelsdorf et al., 1992), or mRXRβ (Mangelsdorf et al., 1992) were constructed and co-transfected with wild type Autographa californiae multiple nuclear polyhedrosis virus DNA into Sf21 cells. Isolation and characterization of recombinant viruses were carried out according to described procedures (O’Reilly et al., 1992).

Polymerase chain reaction technology was used to create restriction sites for the insertion of hRXRα (Mangelsdorf et al., 1996; BspH1, KpnI) and mRXRβ (Mangelsdorf et al., 1992; AflII, KpnI) cDNAs into yeast expression plasmid YepE52, driven by the copper-inducible yeast promoter CUP1 (McDonnell et al., 1988). This plasmid directs synthesis of a yeast receptor fusion protein which is subsequently cleaved by yeast ubiquitinase to release receptor. Both were cloned into an NcoI site immediately 3′ of the reading frame of ubiquitin and a downstream KpnI site. Site-directed mutagenesis was utilized to generate an EcoRI site 5′ to the ATG of mRXRβ (Mangelsdorf et al., 1992) in-frame with the ubiquitin gene. This construct was ligated to a shuttle vector containing the carbosyl-terminal 6 codons of ubiquitin. The AflII/KpnI fragment was ligated into YepE46 (Sone et al., 1990). Oligonucleotides encoding the CRBPII sequence (Mangelsdorf et al., 1991), the RARβ response element (p(RE) sequence (de The et al., 1990; Hoffmann et al., 1990; Sucoff et al., 1990), or a VDRE (Ozono et al., 1991) were designed to include XhoI or Sall restriction site overhangs. These sequences were annealed and subcloned into the XhoI restriction site of the yeast reporter plasmid YRPc2 (Pham et al., 1991).

**Yeasts Strains**—The yeast expression plasmids were transformed into protease-deficient yeast strains BJ5505 (MATα pep4-1A133, prb-1A6R, his3 Iys2-208, trpl-1A01, ura3-32, gln3, can1) and/or BJ2168 (MATα, pep4-3, prb-1-122, pcr-1-407, ura3-32, trpl-1A01) for protein expression together with reporter plasmids into BJ5409 (MATα, leu2-3, his3Δ200, ura3-32, trpl, gal1) for yeast co-transformation assays.

**Protein Extraction Preparation—**Sf21 cells were grown in suspension culture. A density of 2x10⁶/ml with recombinant virus at a multiplicity of infection of 2. Cells were harvested and washed 48 h post-infection by centrifugation at 1000 x g for 10 min at 4 ºC. Cell pellets were resuspended in 2 volumes of lysis buffer (10 mM Tris, pH 7.5, 0.5 mM dithiorethiol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml leupeptin) at 4 ºC and incubated on ice for 1 h. Lysates were produced by using a Dounce homogenizer and a B pestle followed by addition of 2 x 2 ml KC1 to a final concentration of 0.4 M and centrifugation at 100,000 x g for 45 min at 4 ºC to yield the soluble extract.

**Retinoic Acid Binding Assay—**For saturation binding analyses, whole cell high-salt extracts (5–50 µg total protein) were added to 12 x 2 ml vials containing tubes containing 300 μCi [3H]retinoic acid (74 Ci/mmol) and purified at Ligand Pharmaceuticals.

**Antibodies—**Rabbit antiserum against hRARα peptide was kindly provided by J. Dyck (Heyman et al., 1992). We prepared subtype-specific rabbit antisera against hRXRα, mRXRα, hRARβ, mRXRβ, hRARγ, hRARδ, and hRARε, as well as a mouse monoclonal antibody against an hRXRα peptide. A mouse monoclonal antibody against mouse RARβ peptide 423-448 was kindly provided by W. Vedeckis (All et al., 1992). The experiment was performed as described previously (McDonnell et al., 1989) with the following modifications. Growing yeast cultures were diluted to an A₆₀₀ of 0.05–0.50 into 1 ml of 2% yeast extract, 2% peptone, 1% Bacto-agar, and 0.5% dextrose. Yeast cultures were incubated at 200–280 rpm for 12–16 h at 30 ºC with shaking (yeast) or at 37 ºC (CV-1). The cultures were washed and harvested. Lysates were obtained from the incubated cells and media incubated without cells were also extracted in the same manner with ethyl acetate. The extracted material was dried using a Rotovap system prior to resuspension in 0.1 M Tris, pH 7.5, 2 mM EDTA, 50 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 5 mM dithiorethiol, by vortexing with acid-washed glass beads at 4 ºC or via a bead beater (Bioruptor, Joule, Inc., Japan) followed by centrifugation at 100,000 x g for 45 min at 4 ºC to yield the soluble extract.

**RESULTS**

**Hormone Potencies in CV-1 Co-transactivation Assay**—Although 9-cis-RA regulates gene expression through direct binding to RXRα (Heyman et al., 1992; Levin et al., 1992), t-RA is also known to transactivate RXR responsive genes, despite its inability to bind to the RXRs in vitro (Mangelsdorf et al., 1990; Heyman et al., 1992; Allenby et al., 1993). Therefore, the ability of t-RA to transactivate through the RXRs is apparently an indirect effect. To further characterize the biochemical properties of the retinoid acid isomers with members of the RAR and
RXR subfamilies, transcriptional activation studies were performed in a mammalian cell-based assay system.

CV-1 cells co-transfected with a retinoid receptor expression vector and a corresponding luciferase reporter plasmid display a ligand-dependent increase in reporter activity. The transactivation potencies or EC$_{50}$ values (concentrations which give 50% of the efficacy at 10$^{-5}$ M retinoid) for 9-cis-RA and t-RA via the retinoid receptors are summarized in Table I. t-FU acts as described under “Experimental Procedures.” Prestained molecular weight markers (Bio-Rad, 18,500, 49,500, 106,000) were utilized to confirm the production of intact receptors. In control extracts (made from wild-type virus-infected CV-1 cells and probed with RAR subtype-specific antibodies (Fig. 4, lanes 1, 3, 5) there is also lower molecular weight that are not present in the null extracts.

Receptor-Hormone Binding Properties and Comparison to Hormone Transactivation Potencies—To characterize the binding of 9-cis-RA to baculovirus-derived RARs and RXRs, we performed saturation binding analyses with 9-cis-[${^3}$H]RA. A typical binding curve and Scatchard plot for 9-cis-RA binding to RXRs are shown in Fig. 2A. Scatchard analyses of the binding of 9-cis-[${^3}$H]RA to RXRs, -p, -y yield K$_d$ values of 1.62 nm, 2.36 nm, and 2.29 nm, respectively (Table II). We conclude that 9-cis-RA binds to the RXRs with high affinity in a saturable and specific manner. The K$_d$ value obtained here for RXRs is 8-fold lower than our previously reported value of 11.7 nm, measured under different conditions (Heyman et al., 1992), or for other reported values of 9.5–18.3 nm (Levin et al., 1992; Allenby et al., 1993). In the present study, a modification (inclusion of the detergent CHAPS) has led to an improvement in the binding assay by significantly reducing the amount of non-specific binding of 9-cis-RA. These low nanomolar values are more in accord with the higher affinities traditionally observed with the intracellular hormone receptors for their cognate ligands.

In addition to binding to the RXRs, 9-cis-RA also binds to all three members of the RAR subfamily with high affinity (Fig. 2B; Table II). Scatchard analyses of the binding of 9-cis-[${^3}$H]RA to RARa, -p, and -y yield K$_d$ values of 0.31 nm, 0.20 nm, and 0.78 nm, respectively. These data indicate that 9-cis-RA binds with a higher affinity to the RARs than to the RXRs. Our values are almost identical with those determined by Allenby et al. (1993) for the binding of 9-cis-RA to COS-1 cell-expressed RARs. Therefore, the RARs as a group show statistically significant lower dissociation constants for 9-cis-RA (K$_d$ = 0.2–0.8 nm) than do the RXRs for 9-cis-RA (K$_d$ = 1.6–2.4 nm). These binding affinities correlate with the observation that 9-cis-RA is more potent in transactivation of the RARs a, p, and y (EC$_{50}$ = 191 nm, 51 nm, and 45 nm, respectively) than with RXRs, -p, and -y (EC$_{50}$ = 253 nm, 221 nm, and 147 nm, respectively) in the CV-1 assay (Table I). However, since 9-cis-RA is able to bind to both RARs and RXRs, the endogenous complement of these recep-

### Table I

| Hormone | RARa | RARp | RARy | RXRa | RXRp | RXRy |
|---------|------|------|------|------|------|------|
| t-RA    | 352 ± 31 | 82 ± 9 | 10 ± 2 | 916 ± 72 | 1492 ± 134 | 1130 ± 95 |
| 9-cis-RA| 191 ± 20 | 51 ± 17 | 45 ± 5 | 253 ± 41 | 221 ± 29 | 147 ± 13 |

*Values are mean ± S.E. for each value. CV-1 cell co-transfection assays were performed as per Heyman et al. (1992). Each value represents at least 25 individual experiments done in triplicate which were normalized with an internal co-transfected β-galactosidase plasmid.
In addition, although RARγ exhibited a statistically significant weaker binding affinity for 9-cis-RA than did the other RARs (Table II), 9-cis-RA was not less potent with RARγ (EC50 = 45 nM) than with RARα (EC50 = 191 nM) or RARβ (EC50 = 51 nM) in the CV-1 assay (Table I). These data indicate that the binding affinities of 9-cis-RA for the various RAR subtypes do not parallel their transactivation potential values in the CV-1 cell assay (Table II). The presence of other factors in CV-1 cells, such as endogenous retinoid receptor subtypes and/or receptor subtype-specific transcription factors, may contribute to the various levels of activation that are observed with 9-cis-RA via the RARs.

Retinoid Receptor Expression and Transactivation in Yeast —Mammalian cells contain endogenous receptors and factors that promote receptor heterodimer formation which is known to modulate the transcriptional properties of the retinoid receptors. Therefore, we examined the biochemical and transcriptional properties of the RXRs in yeast, a system that lacks intracellular receptors (McDonnell et al., 1989, 1991) and is apparently devoid of factors known to favor receptor heterodimer formation. The RXRs were expressed in yeast strains BJ5409, BJ3505, and BJ2168, and their level of expression and ligand binding dissociation constants for 9-cis-RA were determined. The RXRs expressed in yeast are intact proteins, as analyzed by Western blot analysis (Fig. 1B). The receptor expression levels are 0.1–0.5% of the total soluble yeast protein, depending on yeast strain. The yeast-expressed RXRs have DNA binding properties indistinguishable from those expressed in Sf21 or mammalian cells (data not shown). The yeast-expressed RXRs α, β, and γ bind 9-cis-RA with mean Kd values of 1.44 nM, 1.86 nM, and 1.46 nM, respectively, which are similar to those obtained from Sf21 RXRs (Table II). Quantitation of receptor-hormone binding indicates that 30–60% of the total soluble yeast receptor is ligand binding-competent. A similar percentage of receptor expressed in Sf21 cells is capable of binding hormone.

To examine the transcriptional properties of the RXRs in yeast, the yeast strain BJ5409 was co-transformed with an RXR subtype along with a β-galactosidase reporter construct containing an RXR response element derived from the cellular retinol binding protein II (CRBPII) promoter (Mangelsdorf et al., 1991) linked to the yeast cyc1 promoter (McDonnell et al., 1989). The addition of 9-cis-RA to these cells results in a concentration-dependent increase in β-galactosidase activity for each RXR subtype, with a maximal response of 5–10-fold occurring at 10–6 M and an EC50 of approximately 50–150 nM for each subtype (Fig. 3A). These EC50 values obtained in yeast are similar to those obtained for 9-cis-RA acting through the RXRs in CV-1 cells (Table I). 9-cis-RA does not elicit an activation response when the reporter plasmid was transformed into yeast in the absence of a reporter construct (Fig. 3, A–C), indicating that endogenous RXRs are not present in yeast and that this response is receptor-dependent. The RXRs also activate gene expression in yeast in response to 9-cis-RA via two copies of the BRE (βRE(2)) (Fig. 3C) and via one copy of the βRE (βRE(1)) (Fig. 4A), a retinoid response element derived from the promoter of the RARβ gene (de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990) with efficiencies and potencies similar to that seen with the CRBPII promoter (Fig. 3A). Importantly, the addition of 1 μM 9-cis-RA (or t-RA) to BJ3505 yeast cultures does not increase the RXR (or RAR) protein concentration in the resultant extracts, as determined by Western blot analysis (data not shown) as has been observed for VDR in yeast after the addition of 1,25-dihydroxyvitamin D₃ (Sone et al., 1990). Thus, the observed increase in β-galactosidase activity following the addition of 9-cis-RA to the yeast in the in vivo assays is due to a ligand-dependent transcriptional
from St21 cells transfected without receptor DNA showed no or very low amounts of induced reporter expression vectors into yeast strain BJ5409. Cultures were grown and transformed without receptor DNA. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids. Extracts from yeast transformed with t-RA for 24 h, 9-cis-RA was detected within the cell (Heyman et al., 1992; Levin et al., 1992). This conversion could be enzymatic, and the inability of t-RA to activate the CRBPII-containing reporter via the RXRs in yeast might indicate that yeast do not isomerize t-RA to 9-cis-RA. To test this hypothesis, we incubated CV-1 cells, yeast, or media without cells, with t-RA or 9-cis-RA under conditions identical with those in the transcriptional assays (see "Experimental Procedures"). The composition of retinoic acid isomers present in the organic-extracted material from each source (cell pellet or media) was determined via reversed-phase HPLC monitored at 350 nm for the presence of retinoids (Thaller and Eichele, 1990). Hormone-treated yeast media or CV-1 media without cells shows 5–9% thermal- and/or photoinduced conversion of either added retinoid (Table III and Fig. 5, C and F). Only 3–4% of t-RA is converted to 9-cis-RA in CV-1 cells after 40 h, and approximately 15–20% of the t-RA is converted to other metabolites including 13-cis-RA in both CV-1 cell extracts and in CV-1 media without cells (Table III). Therefore, under the experimental conditions employed here, the conversion of t-RA to other retinoids in CV-1 cells can be accounted for by processes that are not dependent on cellular components. Yeast also show a similar conversion (3–7%) of t-RA to 9-cis-RA when t-RA is incubated either in yeast media alone or in yeast cells (Table III).

**TABLE II**

| t-RA | RARα | RARβ | RARγ | RXRα | RXRβ | RXRγ |
|------|------|------|------|------|------|------|
| **kΔ** |      |      |      |      |      |      |
| 9-cis-RA          | 0.37 ± 0.13 | 0.37 ± 0.13 | 0.22 ± 0.11 |     |      |      |
| Yeast          | 0.31 ± 0.07 | 0.20 ± 0.09 | 0.78 ± 0.14 | 1.62 ± 0.34 | 2.36 ± 0.81 | 2.99 ± 0.84 |

* Dissociation constants (KΔ) were determined by Scatchard analysis. Numbers represent the mean ± S.D. of at least three individual experiments done in duplicate.

† ND indicates no measurable binding detected.

‡ NB indicates value not determined. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids. Extracts from St21 cells transfected without receptor DNA showed no or very low amounts of induced reporter expression vectors into yeast strain BJ5409. Cultures were grown and transformed without receptor DNA. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids. Extracts from yeast transformed without receptor DNA showed no or very low amounts of induced reporter expression vectors into yeast strain BJ5409. Cultures were grown and transformed without receptor DNA. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids. Extracts from yeast transformed without receptor DNA showed no specific binding to retinoids.
and Fig. 8, A and D), indicating a nonenzymatic conversion. It should be noted that while the ratios of t-RA to 9-cis-RA are the same in yeast or CV-1 media or cells, the absolute concentrations of the retinoids in the cells may be different.

Interestingly, 9-cis-RA is more efficiently converted to t-RA in CV-1 cells (25%) than in CV-1 media alone (9%), suggesting the likelihood of an enzymatic process. In yeast, however, there is no statistically significant conversion of 9-cis-RA to t-RA over media controls, indicating that while CV-1 cells may have an enzyme(s) necessary for this conversion, yeast may not have this protein.

**DISCUSSION**

The recent identification of the RAR and RXR subfamilies has provided evidence for distinct and overlapping pathways whereby retinoids exert their broad spectrum of biological activities. An understanding of the RAR and RXR systems requires a knowledge of both their ligand specificities and target gene specificities. We have focused on examining the ligand binding and transcriptional activation properties of two endogenous ligands, 9-cis-RA and t-RA, and have extended our previous observation that 9-cis-RA is a ligand for members of the RXR subfamily (Heyman et al., 1992):

9-cis-RA binds to all members of the RXR subfamily with high affinity, in contrast to the inability of t-RA to bind to the RXRs. The $K_d$ values obtained here for the RXRs are approximately 5-10-fold lower than the previously reported values of 9-18 nM (Heyman et al., 1992; Levin et al., 1992; Allenby et al., 1993). Previously, nonspecific binding of retinoids to cellular extract components has complicated the determination of $K_d$ values for the cellular retinoid binding proteins. In fact, binding assays performed in the past had to incorporate protein separation techniques in order to evaluate the contribution of nonspecific binding (Lotan, 1980; Allegretto et al., 1983). This nonspecific binding has continued to be a problem with nuclear retinoid receptor preparations (Yang et al., 1991; Keidel et al., 1992; Allenby et al., 1993) and, in our hands, is more pronounced with 9-cis-RA than with t-RA. To overcome this problem, we have included a zwitterionic detergent in the hormone binding assay which markedly increases specific binding of retinoids to extract components while decreasing nonspecific binding. The improved $K_d$ values for the RXRs of 1.4-2.4 nM reported here are therefore more in accord with the low nanomolar $K_d$ values of other intracellular receptors for their cognate hormones. More importantly, these values are in a range relevant to the concentration of endogenous 9-cis-RA that has
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Fig. 5. CV-1 and yeast retinoid metabolism. 9-cis-RA (A–C) or t-RA (D–F) was added to yeast (A and D) or CV-1 cell (B and E) cultures or to CV-1 media without cells (C and F) for 16 h at 30 °C (A and D) or 40 h at 37 °C (B, C, E, and F). Washed cells or media were extracted with ethyl acetate and chromatographed via a C18 HPLC column with an isocratic mobile phase. Absorbance of 350 nm was monitored to detect retinoid metabolites (see “Experimental Procedures”). The presence of 350 nm absorbing material was not detected outside the indicated retention time range of 40 to 75 min.

been detected in target tissues (Heyman et al., 1992). These data imply that sufficient endogenous ligand is present in certain tissues to modulate the activity of the RXRs.

We have demonstrated previously in CV-1 co-transfection assays that 9-cis-RA activates not only RXRa, but also RARa (Heyman et al., 1992). Unexpectedly, the present experiments indicate that 9-cis-RA not only binds to the RARs, but does so with greater affinity than to the RXRs. These data are consistent with the observation that 9-cis-RA is more potent in stimulating transcription via RARβ and RARγ than through the RXRs in CV-1 cells. Thus, 9-cis-RA appears to be a “bifunctional” ligand in that it binds and functions as a potent transcriptional activator of both retinoid subfamilies. 9-cis-RA and t-RA both bind equally well to the RARs and induce the transactivation potential of the RARs, implying that both naturally occurring retinoid isomers function to regulate RAR-mediated pathways. The case of an intracellular receptor binding with equal affinities to both a hormone and its naturally occurring metabolite is unprecedented in the steroid receptor field. Whether the dual high affinity interactions of the RAR subtypes with 9-cis-RA and t-RA are physiologically relevant will require further investigation into their circulating levels, stability, and metabolism. These data imply that the metabolism and/or interconversion between t-RA and 9-cis-RA in different target tissues may help dictate which gene pathways are regulated.

Examination of the transcriptional properties of the retinoid receptors in a mammalian cell-based assay system is complicated by the fact that most, if not all, mammalian (and insect) cells contain endogenous retinoid and/or other intracellular receptors. Mammalian and insect cell extracts have been shown to biochemically complement many of the receptors in receptor-specific DNA binding assays (Burnside et al., 1990; Sone et al., 1991; Yang et al., 1991). The RXRs are known to heterodimerize with at least six other known receptors (VDR, two subtypes of TR, and the three RARs; Yu et al., 1991; Leid et al., 1992b; Zhang et al., 1992a; as well as with orphan receptors (Kliewer et al., 1992b, 1992c). Moreover, the Drosophila homologue of RXR, ultraspireacle, can also pair with several of these intracellular receptors (Oro et al., 1992). For these reasons, it is difficult to directly correlate in vitro ligand binding affinities with their ex vivo transactivation properties
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in mammalian or insect systems. In an attempt to avoid this complicating issue, we examined the transactivation properties of the RXRs in yeast, an organism that does not appear to exhibit retinoid receptor activity or activity of any other intracellular receptors (McDonnell et al., 1989, 1991) and does not contain factors that complement intracellular receptors in HRE-DNA binding assays. Importantly, the activity of glucocorticoid receptor, estrogen receptor, and other receptors has been reconstituted in yeast, indicating that fundamental coupling to the transcriptional machinery is conserved in yeast (Schena and Yamamoto, 1988; Metzger et al., 1988).

We show here that 9-cis-RA stimulates transactivation of the CRBPII response element (DR1)-containing promoter in an RXR-dependent manner in yeast with potencies similar to those seen in CV-1 cells. In contrast, t-RA did not stimulate transactivation of this promoter via the RXRs in this yeast system. Thus, in yeast there is a direct correlation between ligand-receptor binding and ligand-induced transactivation; a correlation that is not apparent in mammalian cells. Interestingly, 9-cis-RA also induces transcriptional activation of a βRE(DR5)-containing promoter (but not a VDRE(DR3)) in the presence of RXR. This indicates that RXRs require a specific nucleotide spacing between the half-sites of the direct repeat, implying that they function as homodimers. It is clear from our data that the RXRs function alone in yeast in response to 9-cis-RA, most likely by forming homodimers on promoter elements. These data corroborate and extend the study by Zhang et al. (1992b) that indicates that 9-cis-RA can elicit RXR homodimer formation as assayed by electromobility shift assays, and that the RXRs activate the βRE in insect Schneider cells (a cell type also devoid of RARs), in the presence of 9-cis-RA (our data not shown).

We also show evidence that RARα transactivates via the βRE in yeast, in response to 9-cis-RA or t-RA. The heterodimeric RAR/RXR combination gives an enhanced basal level activity which is further induced when both receptors are occupied by ligand. These functional data from the retinoid receptor promoters, together with the information that yeast do not contain intracellular receptors, indicate the usefulness of yeast as a system of choice for receptor expression and for use as a null receptor background to study the mechanism of receptor transactivation. It will be interesting to test other retinoid compounds in yeast co-transformed with the other RAR subtypes and RAD/RXR combinations via various promoters to further examine the mechanism of retinoid action.

The fact that t-RA does not activate the RXRs in yeast as it does in CV-1 or Schneider cells (Mangelsdorf et al., 1990; Heyman et al., 1992) does not seem to be due to substantial differences in the metabolism of t-RA to 9-cis in mammalian cells versus yeast. In fact there is the same, small percentage of conversion of t-RA to 9-cis-RA in all of the mammalian or insect cells that we have studied, as well as in yeast, or from media without cells. Thus, under the experimental conditions employed here, while 9-cis-RA is present in these cell or media sources at low levels, it does not appear to be generated from t-RA by a cell-dependent enzymatic process. Although the ratio of 9-cis-RA to t-RA is similar in yeast and CV-1 cells, 9-cis-RA may be present in higher concentrations in CV-1 cells, which would explain why the addition of t-RA stimulates transcription via the RXRs in CV-1 cells, but not in yeast. The ability of t-RA to transactivate via the co-transfected RXRs in CV-1 cells may also be modulated by endogenous RARs (absent in yeast) which may bind t-RA (or converted 9-cis-RA) and heterodimerize with RXRs to stimulate these promoters. Additionally, other factors present in CV-1 cells (but absent in yeast) may help modulate the response of RXRs to t-RA. Also, yeast may not be as efficient in the uptake of t-RA versus 9-cis-RA or as compared with CV-1 or Schneider S2 cells and may even exclude retinoids from the cells as has been shown with estrogen in yeast (Gilbert et al., 1993).

While no cell-dependent conversion of t-RA to 9-cis-RA is evident in CV-1 or yeast cells (or in HepG2 or Schneider S2 cells, our data not shown), mammalian cells do exhibit cell-dependent conversion of 9-cis-RA to t-RA. These data may indicate that t-RA is more stable than 9-cis-RA or that an enzymatic pathway that converts 9-cis-RA to t-RA may exist in the cultured cells that we have studied, while the reverse reaction is less favored. While enzymatic conversion of t-RA to 9-cis-RA is not evident in any of the cell lines described here, it is possible that this activity is lost in cultured cells. The 25-hydroxyvitamin D₃-1-a-hydroxylase enzyme that converts 25-hydroxyvitamin D₃ to its active metabolite, 1,25-dihydroxyvitamin D₃ is located in the kidney (Fraser and Kodicek, 1973), but not in most cultured cells, including a variety of kidney cell lines. It will be necessary to examine endogenous retinoid levels in tissues and serum, as well as metabolism in tissues, to gain a better understanding of the normal circulating levels and metabolism of retinoid isomers.

The complexity of the mechanism of action of retinoids is quite apparent. There are at least six subtypes and several isoforms of the nuclear retinoid receptors and the possibility for various combinations thereof in different cell types. The presence and availability of 9-cis-RA and t-RA will determine which receptors are activated, whether homodimers or heterodimers are formed, and ultimately which genes' expression levels are regulated and to what extent. Moreover, different cells may exhibit unique or favored metabolic schemes for interconverting retinoids, depending on the enzyme(s) that they produce. Different cell types may also vary in their pools of transcriptional accessory proteins which may display different specificities for the various retinoid receptor subtypes. In addition, the cellular complement of CRBPs and CRABPs and their affinities for various retinoid metabolites will also determine availability and effective concentration of the ligands. For example, t-RA binds to recombinant CRABP1 with an apparent Kᵩ of 6 nM, while 9-cis-RA does not display saturable binding to CRABP1 (data not shown). Allenby et al. (1993) have also shown recently that while t-RA binds to CRABP1 and -II, 9-cis-RA does not. CRABP overexpression in cells that are normally responsive to t-RA renders them less sensitive to t-RA treatment (Boylan and Gudas, 1992), presumably because the ligand is less available for nuclear receptor uptake. Likewise, 9-cis-RA may be more accessible to the nuclear retinoid receptors if it does not bind to a cellular binding protein. Additionally, retinoid movement through serum to other cells via serum retinoid binding proteins (RBPs) and/or serum albumin may play a role in the function of 9-cis-RA. The fundamental question of whether 9-cis-RA can be transported in serum or whether it is restricted to metabolizing cells must be answered. These and other questions regarding retinoid metabolism and mechanism of action await further study.

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