RESEARCH COMMUNICATION

9-*cis*-Retinoic acid is a natural antagonist for the retinoic acid receptor response pathway

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The pleiotropic activities of retinoids are mediated by two types of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). All-*trans*-retinoic acid (RA) transcriptionally activates RARs, but not RXRs, whereas its natural stereoisomer, 9-*cis*-RA, is the ligand for RXRs. Here, we demonstrate that 9-*cis*-RA did not transcriptionally activate RARs, whereas in the presence of all-*trans*-RA the transactivation of RARs was inhibited in a dose-dependent manner by 9-*cis*-RA. RAR homodimer complexes were destabilized *in vitro* in the presence of 9-*cis*-RA. This suggests that 9-*cis*-RA may be a natural antagonist of all-*trans*-RA for binding to RAR complexes. The levels of 9-*cis*-RA may determine by which pathway the transcription of retinoid-responsive genes is modulated.

INTRODUCTION

Retinoids are derivatives of retinol (vitamin A) that have profound effects in embryonic development, cellular differentiation and proliferation [1,2]. They are successfully used for the treatment of skin diseases and are promising anti-cancer drugs [3]. The cellular receptors for retinoids, retinoic acid receptors (RARs) [4–8] and retinoid X receptors (RXRs) [9–11], are members of the steroid-receptor superfamily [12,13] that are ligand-induced transcription factors. RARs were believed to act exclusively as heterodimers with RXRs [14–19], whereas RXRs were able to act also as homodimers [20,21]. However, RXR-independent transactivations and gel shifts with RARs translated *in vitro* [22] also indicated the existence of RAR homodimers, although an ultimate proof for their physiological importance has not been presented. Taken together, there seem to exist three different retinoid-receptor complexes with overlapping but not identical response-element specificity. The relative amounts of RARs and RXRs are probably one way to determine which of the three pathways regulates retinoid-inducible genes. An additional regulatory level is the modulation of the receptors by their specific ligands. The natural ligand of RXRs, 9-*cis*-RA, is also bound *in vitro* with high affinity by RARs [23,24], whereas all-*trans*-RA has only affinity to RARs. Due to partial isomerization and metabolism during transient transfections, all-*trans*- and 9-*cis*-RA may not show pure effects on receptor activation.

Here, we investigate the effects of all-*trans*- and 9-*cis*-RA on transactivation and DNA binding affinity of RAR–RXR heterodimers, RXR homodimers and RAR homodimers under experimental conditions that diminish isomerization.

MATERIALS AND METHODS

DNA constructs

The RA response element of the RARβ gene promoter [25] was subcloned into the *Xba*I site of pBLCAT2 [26] upstream of the *tk* promoter driving the expression of the chloramphenicol acetyltransferase (CAT) gene (compare [22]). RARα and RXRα cDNAs subcloned into pSG5 (Stratagene) were kindly provided by J. F. Grippo (Hoffmann–LaRoche, Nutley, U.S.A.).

Cell culture, transfection and CAT assays

SL-3 cells (5×10^5 cells per well on a 6-well plate) were grown overnight in Schneider's medium supplemented with 15% (v/v) charcoal-treated fetal-calf serum (FCS). For transfection, liposomes were formed by incubating 1 μg of reporter plasmid, 0.25 μg of expression plasmid for RARα and/or RXRα, and 1 μg of the reference plasmid pCH110 (Pharmacia) with 15 μg of *N*-[1-(2,3-Dioleoloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) (Boehringer-Mannheim) for 15 min at room temperature in a total volume of 50 μl. After dilution with 1 ml of Schneider's medium the liposomes were added to the cells and 1 ml of Schneider's medium, supplemented with 30% (v/v) charcoal-treated FCS, was added 8 h after transfection. At this time the ligand or solvent was also added. The actual concentration of retinoids in the supernatant was calibrated with ^{3}H-labelled all-*trans*-RA. The cells were harvested 16 h after initiation of the treatment (if not otherwise indicated).

CV-1 cells (5×10^5 per 60-mm diam. Petri dish) were grown overnight in Phenol Red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) charcoal-treated FCS. Liposomes were formed by incubating 5 μg of the reporter plasmid, 2 μg of expression plasmid for RARα and/or RXRα, and 3 μg of pCH110 with 30 μg of DOTAP for 15 min at room temperature in a total volume of 100 μl. After dilution with 1.4 ml of Phenol Red-free DMEM, the liposomes were added to the cells and 8 h later 1.5 ml of Phenol Red-free DMEM supplemented with 20% (v/v) charcoal-treated FCS together with ligand or solvent were also added. The cells were harvested 8 h after initiation of the treatment.

CAT assays were performed as described [27]. Protein concentrations were determined by the Bradford method (Bio-Rad) and transfection efficiency was determined by β-galactosidase activity.

Abbreviations used: RARs, retinoic acid receptors; RXRs, retinoid X receptors; RA, retinoic acid; CAT, chloramphenicol acetyltransferase; *usp*, ultraspiracle gene; FCS, fetal-calf serum; DOTAP, *N*-[1-(2,3-Dioleoloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate; DMEM, Dulbecco's modified Eagle's medium.

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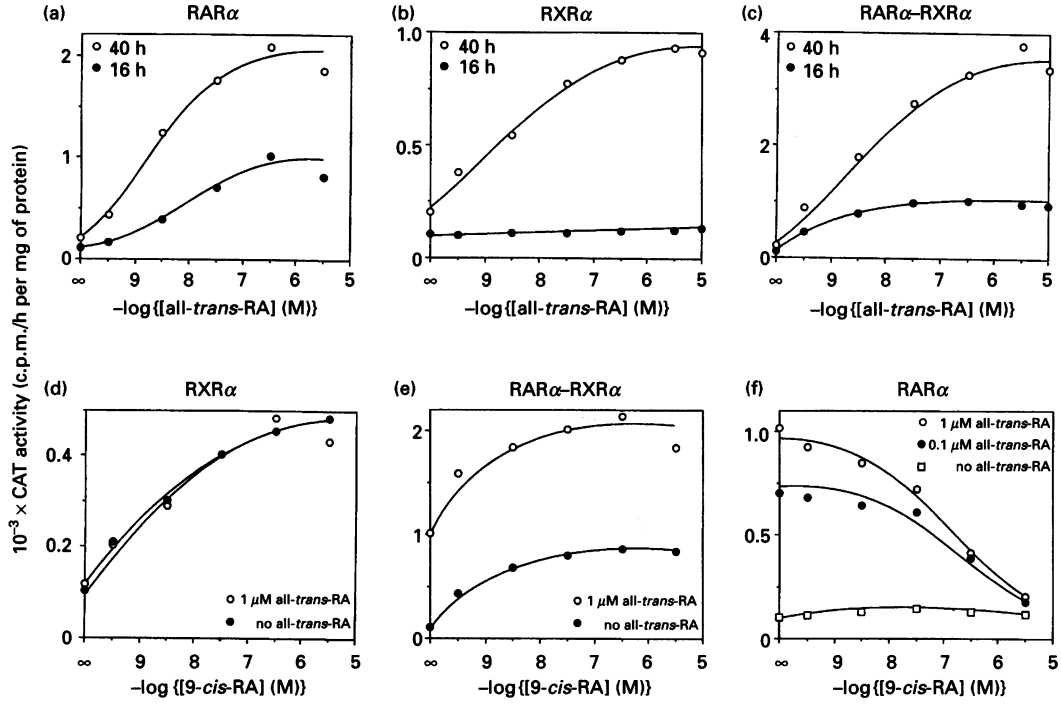

Figure 1 Dose response to all-*trans*- and 9-*cis*-RA of RAR and RXR transactivation in SL-3 cells

Cells were transfected with the βRARE–*tk*–CAT reporter construct [9] and with expression vectors for RARα (**a**, **f**), for RXRα (**b**, **d**) or for both (**c**, **e**). The cells were treated with increasing concentrations of all-*trans*-RA (**a**–**c**) or 9-*cis*-RA (**d**–**f**). Dose responses to 9-*cis*-RA were in the presence of indicated concentrations of all-*trans*-RA. CAT activities were determined 16 h later (after 40 h, as indicated in **a**–**c**). Each data point represents the mean of triplicates; S.D. was always < 10%.

In vitro translation of retinoid receptors and DNA-binding assays

Linearized cDNAs for RARα and RXRα were transcribed *in vitro* as recommended by the supplier (Promega). Samples (5 μg) of each RNA were mixed with 175 μl of rabbit reticulocyte lysate, 100 units of RNasin and 20 μM complete amino acid mixture (all from Promega) in a total volume of 250 μl and incubated at 30 °C for 180 min. The RA response element (βRARE) probe was prepared by double digestion of the respective reporter plasmid with *Hin*dIII and *Bam*HI, purification by gel electrophoresis and labelling by a fill-in reaction using [α-^{32}P]dCTP and T7 DNA polymerase (Pharmacia). Samples (5 μl) of receptors translated *in vitro* were preincubated with ligand for 10 min at room temperature in a total volume of 20 μl of binding buffer [10 mM Hepes (pH 7.9), 80 mM KCl, 1 mM dithiothreitol, 0.2 μg/μl poly(dI/dC) and 5% (w/v) glycerol]. Then about 1 ng of labelled probe (25000 c.p.m.) was added and the incubation was continued for 30 min. The protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel at room temperature in 0.5 × TBE (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.3) and a Kodak XAR film was exposed to the dried gel.

RESULTS AND DISCUSSION

The study of the activation of an individual retinoid receptor in mammalian cells is hampered by the fact that most of them express high levels of both RARs and RXRs. We found that *Drosophila* SL-3 cells [28] are an appropriate system: (i) they are devoid of endogenous RARs and RXRs, (ii) they do not respond to retinoids, and (iii) they seem not to express sufficient amounts of endogenous proteins that might be capable of forming heterodimers with RARs [22], such as the ultraspiracle (*usp*) gene product [29–31]. Thus the three retinoid response pathways could be reconstituted individually by transfecting SL-3 cells with selected receptor expression plasmids together with the RA response element of the human RARβ2 promoter [25] driving the expression of the CAT reporter gene. We studied the transcriptional activation of these pathways by dose–response curves to all-*trans*- and 9-*cis*-RA. The responses of RARα (Figure 1a), of RXRα (Figure 1b) or of both together (Figure 1c) to all-*trans*-RA after 40 h of stimulation reached a plateau at about 1 μM, corresponding to about 10-, 5- and 20-fold induction of CAT activity respectively. On shortening stimulation time to reduce isomerization of retinoids, only RARα- and RARα–RXRα-transfected cells responded to all-*trans*-RA (Figures 1a and 1c) and yielded for both a stimulation of about 10-fold, whereas cells expressing RXRα showed no significantly increased CAT activity (Figure 1b). These results confirm previous observations [11,23] that all-*trans*-RA does not bind and activate RXRs. The observed activation of RXRs at 40 h of stimulation may be, therefore, due to partial isomerization of all-*trans*-RA into 9-*cis*-RA. Consequently, for further experiments only short-term stimulations (16 h) were applied.

The dose–response curve of RXRα induced by 9-*cis*-RA showed saturation of CAT activity at 1 μM (Figure 1d), and even the co-presence of 1 μM all-*trans*-RA provided the same results. This confirms that there was no isomerization from all-*trans*-RA into 9-*cis*-RA. The transactivation of RARα–RXRα by 9-*cis*-RA showed saturation at 0.5 μM (Figure 1e). In the presence of 1 μM all-*trans*-RA the stimulation was increased from 10-fold (in the absence of all-*trans*-RA) to 20-fold, confirming our previous

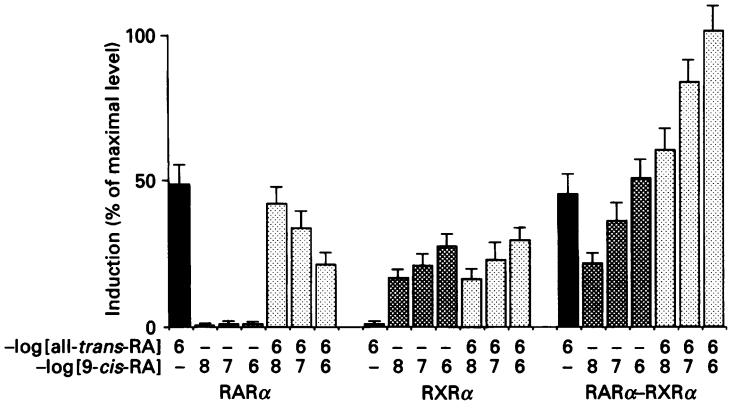

Figure 2 Ligand-dependent transactivation of RARs and RXRs overexpressed in CV-1 cells

Cells were transfected with the βRARE–*tk*–CAT reporter construct and with the expression vectors for RARα, for RXRα, or for both, as indicated. The transfected cells were treated for 8 h with the indicated ligand concentrations. The columns indicate the means of triplicates and the bars are S.D.s.

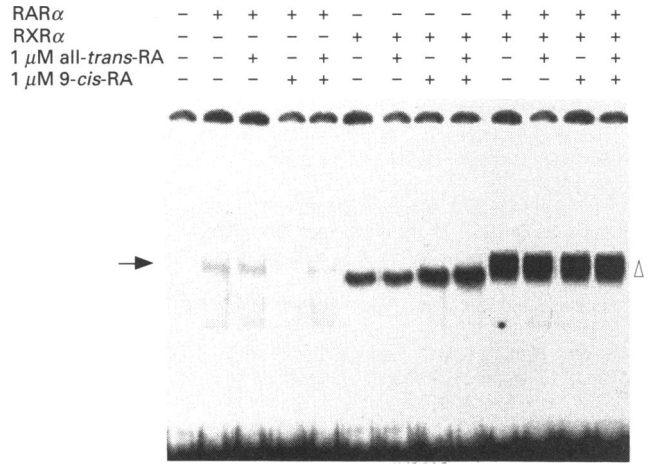

Figure 3 Analysis of the ligand-dependent binding of RARs and RXRs translated *in vitro*

A band-shift analysis was performed using RARα and RXRα translated *in vitro* (preincubated with 9-*cis*-RA, all-*trans*-RA or solvent, as indicated) and the βRARE probe. The specificities of the shifted bands were proved by comparison with unprogrammed lysate (first lane). The triangle indicates RXR homodimers with slightly higher mobility than RAR homo- and heterodimers (arrow).

observation [22] that RAR–RXR-driven gene activity is maximal in the presence of both ligands.

Interestingly, RAR-driven gene activity was not induced by 9-*cis*-RA (Figure 1f), although this isomer binds to RARs with the same affinity as all-*trans*-RA [32]. This also indicates that under these experimental conditions there is little or no isomerization of 9-*cis*-RA into all-*trans*-RA. Furthermore, the CAT activity of all-*trans*-RA-induced RARs was decreased in a dose-dependent manner by 9-*cis*-RA, showing an EC_{50} of about 100 nM. The antagonistic effect of 9-*cis*-RA was also tested in African Green Monkey kidney (CV-1) cells that show only low endogenous expression of retinoid receptors. We found that induction time should not extend over 8 h to sufficiently diminish isomerization of the ligands in this cell line (results not shown). Stimulations with different retinoid concentrations were performed after co-transfection of expression vectors for RARα, for RXRα, or for both. Experiments performed in parallel with co-transfected parental expression vector provided a background of endogenous receptor activity, which was subtracted from that derived with overexpressed receptors. Figure 2 shows the respective stimulation factors. The all-*trans*-RA-induced activity of overexpressed RARα was decreased in a dose-dependent manner by 9-*cis*-RA. This reproduced the antagonistic effect of 9-*cis*-RA in the mammalian system. The diminished isomerization was demonstrated by the inability of all-*trans*-RA to induce overexpressed RXRα. As also observed in the *Drosophila* system, CV-1 cells, transfected with both RARα and RXRα, showed increased stimulation when exposed to both ligands than with either alone.

In a similar experimental system [20] all-*trans*-RA, as well as 9-*cis*-RA, was reported to be an inducer of RARs, which was probably due to isomerization by long-term stimulation, as two stable analogues of 9-*cis*-RA showed nearly no induction. The computer modelling of these two compounds demonstrated their close similarity to the 9-*cis*-RA structure, suggesting that they bind to RARs also, which supports our finding of the antagonistic effect of 9-*cis*-RA.

In order to study the effects of ligands on DNA binding *in vitro* we performed gel-shift experiments with RARs and RXRs in the presence or absence of the two retinoids (Figure 3). RARα translated *in vitro* yielded a specific but weakly retarded complex that was not affected by all-*trans*-RA, whereas 1 μM 9-*cis*-RA decreased its stability. In the presence of all-*trans*-RA, 9-*cis*-RA weakens this complex. The position of the complex in the gel is nearly identical to that of RXR homodimers or RAR–RXR heterodimers (compare also [22]). Since RARs do not bind as monomers to DNA (results not shown), it is very likely that the observed complex represents RAR homodimers and not two independently binding monomers. As reported previously [21], the DNA-binding affinity of RXR homodimers was increased by 9-*cis*-RA but not affected by all-*trans*-RA, whereas the affinity of RAR–RXR heterodimers for DNA was not influenced by the ligands.

Synthetic retinoids have been reported that selectively bind RARα, but do not activate the receptor in a transactivation assay [33]. We found that the natural retinoid 9-*cis*-RA displays similar characteristics to these RARα-selective antagonists in *Drosophila* as well as in the mammalian system. This antagonistic action is in contrast with the role of 9-*cis*-RA as activator of the

two retinoid signalling pathways mediated by RXR homodimers and RAR–RXR heterodimers, and provides further evidence for the *in vivo* relevance of a third pathway that may be mediated by RAR homodimers.

As reported recently [32], 9-*cis*-RA does induce the transactivation of a monomer of a chimeric receptor composed of the GAL4 DNA-binding and the RARα hormone-binding domain, suggesting that binding of 9-*cis*-RA to full-length RARs does not inhibit transactivation. However, the bent structure of 9-*cis*-RA possibly induces a conformational change of the RAR protein core thereby decreasing its ability to dimerize and, therefore, reducing its affinity to DNA.

The dual function of 9-*cis*-RA, either as antagonist for RARs or agonist for RXRs, appears to be a further regulatory mechanism explaining the pleiotropic effects of retinoids in the control of cell growth and differentiation. *In vivo* isomerization of 9-*cis*-RA makes it probably unsuitable for clinical trials; however, its stable synthetic analogues should be of therapeutic interest.

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REFERENCES

1 Thaller, C. and Eichele, G. (1990) Nature (London) **345**, 815–819
2 de Luca, L. M. (1991) FASEB J. **5**, 2924–2933
3 Saurat, J.-H. (ed.) (1990) Retinoids: 10 years on, Karger, Basel
4 Giguère, V., Ong, E. S., Segui, P. and Evans, R. M. (1987) Nature (London) **330**, 624–629
5 Petkovich, M., Brand, N. J., Krust, A. and Chambon, P. (1987) Nature (London) **330**, 444–450
6 Benbrook, D., Lernhardt, E. and Pfahl, M. (1988) Nature (London) **333**, 669–672
7 Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1988) Nature (London) **332**, 850–853
8 Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5310–5314
9 Hamada, K., Gleason, S. L., Levi, B.-Z., Hirschfeld, S., Appella, E. and Ozato, K. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8289–8293
10 Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. and Evans, R. M. (1990) Nature (London) **345**, 224–229
11 Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A. and Evans, R. M. (1992) Gen. Dev. **6**, 329–344
12 Evans, R. M. (1988) Science **240**, 889–895
13 O'Malley, B. W. (1990) Mol. Endocrinol. **4**, 363–369
14 Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Näär, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. and Rosenfeld, M. G. (1991) Cell **67**, 1251–1266
15 Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) EMBO J. **11**, 1409–1418
16 Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. and Evans, R. M. (1992) Nature (London) **355**, 446–449
17 Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) Cell **68**, 377–395
18 Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M. and Ozato, K. (1992) EMBO J. **11**, 1419–1435
19 Zhang, X.-K., Hoffmann, B., Tran, P. B.-V., Graupner, G. and Pfahl, M. (1992) Nature (London) **355**, 441–446
20 Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I. and Pfahl, M. (1992) Science **258**, 1944–1946
21 Zhang, X.-K., Lehmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Herrmann, T., Tran, P. and Pfahl, M. (1992) Nature (London) **358**, 587–591
22 Schräder, M., Wyss, A., Sturzenbecker, L. J., Grippo, J. F., LeMotte, P. and Carlberg, C. (1993) Nucleic Acids Res. **21**, 1231–1237
23 Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992) Cell **68**, 397–406
24 Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J. F. (1992) Nature (London) **355**, 359–361
25 de Thé, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990) Nature (London) **343**, 177–180
26 Luckow, B. and Schütz, G. (1987) Nucleic Acids Res. **15**, 5490
27 Pothier, F., Ouellet, M., Julien, J.-P. and Guérin, S. L. (1992) DNA Cell Biol. **11**, 83–90
28 Schneider, I. (1972) J. Embryol. Exp. Morph. **27**, 353–365
29 Oro, A. E., McKeown, M. and Evans, R. M. (1990) Nature (London) **347**, 298–301
30 Yao, T.-P., Segraves, W. A., Oro, A. E., McKeown, M. and Evans, R. M. (1992) Cell **71**, 63–72
31 Thomas, H. E., Stunnenberg, H. G. and Stewart, A. F. (1993) Nature (London) **362**, 471–475
32 Allenby, G., Bocquel, M.-T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P. and Levin, A. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 30–34
33 Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W. and Klaus, M. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 7129–7133

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