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Retinoid X receptor α forms tetramers in solution

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ABSTRACT **Protein–protein interactions allow the retinoid X receptor (RXR) to bind to cognate DNA as a homo- or a heterodimer and to participate in mediating the effects of a variety of hormones on gene transcription. Here we report a systematic study of the oligomeric state of RXR in the absence of a DNA template. We have used electrophoresis under nondenaturing conditions and chemical crosslinking to show that in solution, RXRα forms homodimers as well as homotetramers. The dissociation constants governing dimer and tetramer formation were estimated by fluorescence anisotropy studies. The results indicate that RXR tetramers are formed with a high affinity and that at protein concentrations higher than about 70 nM, tetramers will constitute the predominant species. Tetramer formation may provide an additional level of the regulation of gene transcription mediated by RXRs.**

Retinoids exert multiple effects on proliferation and differentiation of a variety of mammalian cells. They play a key role in epithelial cell differentiation in fetal and adult organs, have profound effects on limb and nervous system morphogenesis, are potent inhibitors of carcinogenesis in rodent models, and are currently used as chemopreventive and therapeutic agents in several types of cancer (1–4). Signaling by two physiologically active retinoids, all-*trans*- and 9 *cis*-retinoic acids, is mediated by two classes of proteins which belong to the steroid/thyroid/retinoid hormone superfamily of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (5, 6). These receptors bind to regulatory elements upstream from target genes and act as ligand-activated transcription factors.

Three subclasses of RARs and RXRs (α, β, and γ) and several isoforms of each are known to exist (5, 6). RARs bind all-*trans*- as well as 9-*cis*-retinoic acid, whereas RXRs bind only the 9-*cis* isomer (7–9). Retinoid receptors can bind to their response elements, which consist of polymorphic arrangements of the nucleotide sequence motif 5′-RG(G/T)TCA-3′ (10), as homodimers or as RAR/RXR heterodimers. RXRs can also dimerize with other members of the steroid/thyroid/retinoid receptor family, and heterodimerization usually increases the efficiency of the interactions with DNA and the ensuing transcriptional activation (11). Binding of receptor dimers to DNA containing two half-sites of the consensus motif is characterized by a marked positive cooperativity (10), indicating strong protein–protein interactions. Two regions within retinoid receptors have been implicated in governing dimer formation; the main dimerization region, consisting of a heptad repeat motif, is found within the ligand-binding domain, while a weaker dimerization function is found within the DNA-binding domain (10, 12–15).

To date, studies of the self-association of retinoid receptors have been carried out almost exclusively in the presence of DNA templates. It has been reported that retinoid receptors form homo and hetero complexes also in the absence of cognate DNA (16, 17), but, in another study, chemical crosslinking of retinoid receptors failed to demonstrate the existence of species higher than monomers (18). The present study is a systematic investigation of the self-association of a retinoid receptor in solution and it leads to the surprising conclusion that, in the absence of cognate DNA, RXR has a high propensity to form tetramers.

MATERIALS AND METHODS

Protein. RXRα lacking the N-terminal A/B domain (RXR$\alpha\Delta$AB) was obtained by overexpression in *Escherichia coli* essentially as described (19). *E. coli* cells harboring the RXR$\alpha\Delta$AB gene on the pET15b plasmid were grown at 37°C to an OD of 0.6–0.8. After induction with 0.5 mM isopropyl β-D-thiogalactopyranoside, cells were grown for an additional 2.5 hr. Cell lysis and protein purification procedures were followed as described (19) except for the omission of freeze–thaw cycling in cell lysis. Protein concentration was determined by the Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

Oligonucleotides. Oligonucleotides with the RXR consensus response element DR-1, which contains two direct repeats spaced by a single nucleotide (5′-TCGAGGGTAGGGGTCAGAGGTCACTCG-3′), were synthesized and purified at the Cornell Biotechnology Center. Single-stranded DNA was annealed (20) and double-stranded DNA was isolated on Centrex centrifugal filter units (Schleicher & Schuell).

Nondenaturing Gel Electrophoresis. The receptor was electrophoresed under nondenaturing conditions in polyacrylamide gels with various acrylamide concentrations. Electrophoresis was carried out at pH 8.0, 8.8, and 9.5. The mobilities of the bands were analyzed by the method of Ferguson as described by Boilag and Edelstein (21). The retardation factors (R_f values) of individual bands in gels of differing acrylamide concentrations were plotted versus the acrylamide concentration and the resulting slopes were compared with those of protein standards with known molecular weights. For the lane shown in Fig. 1, RXR$\alpha\Delta$AB (50 μM) was resolved in a 10% polyacrylamide gel (pH 8.8) cooled with circulating tap water.

Chemical Crosslinking of RXR$\alpha\Delta$AB. One microliter of various concentrations of disuccinimidyl suberate (DSS) in dimethyl sulfoxide was added to 9 μl of 113 μM RXR$\alpha\Delta$AB in buffer A [10 mM Hepes, pH 8.0/0.1 mM EDTA/0.4 mM dithiothreitol/400 mM KCl/5% (vol/vol) glycerol]. Mixtures were incubated for 15 min at room temperature, and the reaction was terminated by the addition of 3 μl of 1 M ethanolamine. Samples were resolved by SDS/PAGE in 7.5% acrylamide gels cooled with circulating tap water, and protein bands were visualized by staining with Coomassie blue. In control experiments, proteins other than RXR were crosslinked under similar conditions: retinol-binding protein (RBP) displayed one band at 21 kDa, corresponding to RBP monomers, and transthyretin, a protein with a subunit molecular

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Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; DSS, disuccinimidyl suberate.

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mass of 16 kDa that is known to exist as a tetramer, showed protein bands corresponding to monomers and tetramers.

Labeling of RXRαΔAB with a Fluorescent Probe. RXRαΔAB (10–15 μM) in buffer A was covalently labeled with bromoethylfluorescein in dimethylformamide. The mixture was incubated for 2–3 hr at room temperature and dialyzed extensively against buffer A to remove unreacted probe. The stoichiometry of labeling in different preparations was 0.5–2 mol of probe per mol of RXRαΔAB.

Fluorescence Decay Measurements. Fluorescence decays were measured with a time-resolved single-photon-counting instrument with a deuterium flash lamp as an excitation source. The excitation wavelength was selected by a Jobin–Yvon monochromator. Emitted light was collected through a Corning CS 3-70 cutoff filter. The decays were modeled as a single exponential or sum of exponentials and were fit by iterative convolution with the instrument response function by means of a standard nonlinear least-squares program (22).

Fluorescence Anisotropy Titrations. The fluorescence anisotropy (r) of a labeled protein complex is related to its rotational volume through the Perrin equation:

$$r_0/r - 1 = RT\tau/\eta V_r, \quad [1]$$

where r_0 is the anisotropy in the absence of rotational motion, R and T are the gas constant and the absolute temperature, respectively, τ is the fluorescence lifetime, η is the solvent viscosity, and V_r is the rotational volume. In the absence of changes in τ, changes in the fluorescence anisotropy directly report on changes in the rotational volume of a fluorophore—e.g., changes due to macromolecular association (23). To estimate the predicted fluorescence anisotropy changes upon association of the protein to dimers or tetramers, the following values were used: $r_0 = 0.31$ as was measured by standard procedures (23); $\eta = 1.1$ cP; V_r was calculated by taking the partial specific volume of RXR to be 0.74 (based on the amino acid composition). To study the concentration dependence of the fluorescence anisotropy of the labeled RXRαΔAB, the protein was diluted in buffer A containing 100 mM KCl, incubated overnight at 4°C, and equilibrated at 22°C for 30–90 min prior to measurements. The values of the fluorescence anisotropy (excitation, 491 nm; emission, 516 nm) at each point were measured four or five times to obtain a mean. The standard deviation of the measurements was 0.0024 anisotropy unit. Measurements were performed at 22°C with a SPEX Fluorolog 2 spectrofluorometer equipped with Glan–Thompson polarizers.

Analyses of Fluorescence Anisotropy Data. The fluorescence anisotropy titration curves were analyzed with the numerically based program BIOEQS, which allows for analysis of models containing multiple subunit equilibria (24).

RESULTS

The protein used in these experiments, RXRα lacking the terminal A/B domain (RXRαΔAB), possesses a high affinity for 9-*cis*-retinoic acid and retains its ability to properly form dimers, as was shown by the strong positive cooperativity in dimer binding to a consensus response element and by its ability to form heterodimers with RAR (19).

Electrophoresis Under Nondenaturing Conditions. When RXRαΔAB was electrophoresed under nondenaturing conditions at pH 8.8 in a 10% polyacrylamide gel, a weak fast-moving band and two intense slower-moving bands were observed (Fig. 1). Under all conditions, electrophoresis of RXRαΔAB gave rise to the three distinct bands, the relative intensity of which varied with electrophoresis conditions and receptor concentration. However, as can be seen in Fig. 1, the faster moving band was always the faintest and the main variations were observed between the intensities of the two

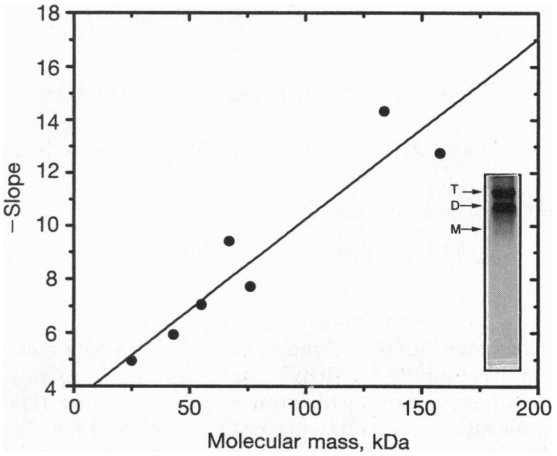

FIG. 1. Gel electrophoresis of RXRαΔAB under nondenaturing conditions. RXRαΔAB and protein standards of known molecular mass (chymotrypsinogen, ovalbumin, transthyretin, bovine serum albumin, transferrin, and aldolase) were electrophoresed under nondenaturing conditions in polyacrylamide gels of various acrylamide concentrations. Retardation factors (R_f values) were measured and plotted as $100 \cdot \log(100 \cdot R_f)$ versus % acrylamide to yield linear relationships. The slopes of lines for the individual protein standards are plotted versus their known molecular mass in the form of a Ferguson plot (24). (*Inset*) Three bands were observed in lanes containing RXRαΔAB. For the lane shown, electrophoresis was carried out with 50 μM RXRαΔAB in a 10% polyacrylamide gel (pH 8.8) cooled with circulating tap water. M, monomer; D, dimer; T, tetramer.

slower bands. Analyses of five experiments at different electrophoresis pH yielded average molecular masses of the three RXRαΔAB species of 42 ± 7, 68 ± 4, and 150 ± 11 kDa (mean ± SEM). As the molecular weight of RXRαΔAB is 40,270 (25), the data are consistent with the presence of RXRα monomers, dimers, and tetramers.

Chemical Crosslinking of RXRαΔAB. To further confirm the composition of RXRαΔAB oligomers, the receptor was covalently crosslinked with the crosslinking reagent DSS and analyzed by SDS/PAGE (Fig. 2*A*). Two protein bands with electrophoretic mobilities corresponding to protein monomer and dimer were observed at low DSS concentrations. At higher concentrations of the crosslinking reagent, a protein band at 160–170 kDa became visible, confirming the presence of tetramers. Similar results were obtained when a sulfhydryl group-attacking crosslinker, *N,N'*-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine, was used (data not shown).

The formation of RXRαΔAB tetramers in solution is surprising because it is well established that the receptor interacts with cognate DNA as a dimer. To assess the oligomerization state of RXRαΔAB when bound to DNA, and to verify that the appearance of tetramers in crosslinked preparations did not originate from nonspecific aggregation, RXRαΔAB was covalently crosslinked in the presence of an oligonucleotide containing the RXR consensus response element DR-1. As the concentration of the oligonucleotide was increased, a shift in receptor species distribution was observed such that protein monomers and dimers became more prominent, whereas the level of tetramers was diminished (Fig. 2*B*). These data suggest that in the presence of cognate DNA, RXRα dimers bind to the oligonucleotides, leading to dissociation of tetramers.

Binding Affinities Within RXRαΔAB Oligomers. To estimate the binding affinities characterizing the self-association of RXRαΔAB, the protein was covalently labeled with fluorescein and the concentration dependence of the fluorescence anisotropy of the labeled protein was examined. Fluorescence anisotropy is a measure of the molecular size of a fluorescent molecule and provides a sensitive tool for studies of reactions leading to changes in the size of a protein complex, such as

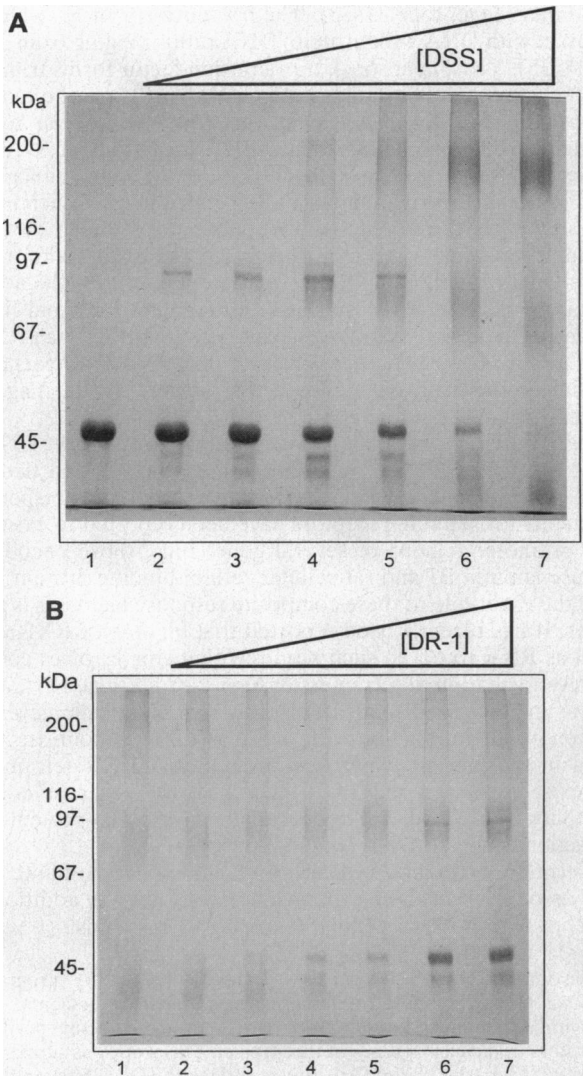

FIG. 2. Chemical crosslinking of RXRαΔAB. (*A*) Crosslinking of RXRαΔAB in solution. RXRαΔAB was crosslinked by DSS and samples were resolved by SDS/PAGE in a 7.5% polyacrylamide gel (pH 8) cooled with circulating tap water. The final concentrations of DSS for lanes 1–7 were 0, 0.025, 0.05, 0.2, 0.5, 1, and 2.5 mM, respectively. (*B*) Crosslinking of RXRαΔAB in the presence of a DR-1 response element. RXRαΔAB (40 μM) was incubated for 6 hr at 4°C in a total volume of 18 μl with an oligonucleotide containing a DR-1 response element. The final concentrations of the oligonucleotide for lanes 1–7 were 0, 2, 5, 10, 20, 35, and 50 μM, respectively.

oligomerization (e.g., refs. 23, 26–28). The fluorescence lifetime of fluorescein-labeled RXRαΔAB at 30 and 600 nM was found to be 7.5 and 7.8 ns, respectively, indicating that it was independent of protein concentration in this range and that changes in anisotropy directly reflected protein oligomerization. The concentration dependence of the fluorescence anisotropy of RXRαΔAB in two independent experiments is shown in Fig. 3. When the protein concentration was increased, an increase in fluorescence anisotropy was observed, reflecting protein association. The anisotropy approached a constant value at high protein concentrations, demonstrating that the association reaction did not reflect nonspecific protein aggregation, but a discrete self-association process with a well-defined end point. To ascertain that the plateau observed at the end of titration curves did not reflect the limiting ability of the fluorescein probe to report on large oligomers, RXRαΔAB was labeled with the longer-lived fluorescent probe pyrenesulfonyl chloride. The fluorescence lifetime of pyrene is 5–6 times longer

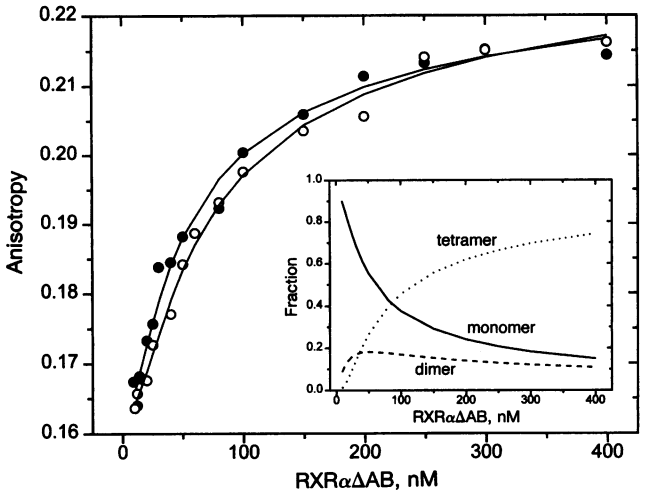

FIG. 3. Fluorescence anisotropy titration of fluorescein-labeled RXRαΔAB. Fluorescence anisotropy of fluorescein-labeled RXRαΔAB at increasing protein concentrations was measured as described in *Materials and Methods.* Results from two independent experiments are shown. The lines represent the fit of the data to a model containing protein monomers, dimers, and tetramers, calculated with the BIOEQS program (see text). (*Inset*) Fractional distribution of RXRαΔAB species as a function of protein concentration. Curves were generated by the BIOEQS program using 9.15 and 29.37 kcal/mol for ΔG°_{dimer} and $\Delta G^{\circ}_{tetramer}$, respectively (see text).

than that of fluorescein and it thus can report on the formation of larger aggregates. The titration curves obtained with pyrene-labeled RXR were almost identical in appearance to those shown in Fig. 3 and yielded association energies that were similar to those reported below (data not shown). To estimate the free energies of formation of RXRαΔAB oligomers, the experimental data were fitted by means of a numerically based computer program capable of analyzing multiple binding equilibria (24). The species that were included in the fitting routine were monomers, dimers, and tetramers. The limiting values of fluorescence anisotropies at the beginning and the end of the titrations were taken to represent protein monomers and tetramers, respectively. The best fits for the data in Fig. 3 were obtained with limiting anisotropies of 0.161 and 0.233 for monomers and tetramers, respectively. The overall anisotropy change during the titration was thus 0.072. Anisotropy changes that are expected to accompany tetramerization of a 40-kDa protein can be estimated from Eq. **1** to be about 0.073 (see *Materials and Methods*). Thus, the data in Fig. 3, though they do not constitute evidence for the presence of RXR tetramers, are consistent with a monomer–dimer–tetramer model.

To set the value of fluorescence anisotropy for dimers, analyses in which this parameter was varied between 25% and 75% of the tetramer–monomer interval were carried out. The recovered ΔG° values for formation of both dimers and tetramers were not sensitive to this parameter and varied by only 1–2% in the tested range. Consequently, the anisotropy for dimers was set at an intermediate value between the limiting anisotropies. Additional uncertainties are associated with the assumption that at the lowest protein concentration used (5–10 nM) the protein existed predominantly as a monomer. However, the goodness of fit was excellent (χ^2 for the curves shown in Fig. 3 was 0.29 and 0.67); in addition, small variations in the set anisotropy value for protein monomers did not affect the free energy of the dimer → tetramer transition, and fits always showed significant positive cooperativity in formation of tetramers versus dimers (see below). The fit for the data from the experiments shown in Fig. 3 yielded mean ΔG° values for formation of dimers and tetramers from RXR monomers of 9.15 and 29.37 kcal/mol of complex, respectively.

The free energy of formation of protein tetramers from protein dimers can be computed from these data by using the following relation:

$$\Delta G^{\circ}_{\text{dimer} \rightarrow \text{tetramer}} = \Delta G^{\circ}_{\text{monomer} \rightarrow \text{tetramer}} - 2 \cdot \Delta G^{\circ}_{\text{monomer} \rightarrow \text{dimer}} \quad [2]$$

Accordingly, the calculated $\Delta G^{\circ}_{\text{dimer} \rightarrow \text{tetramer}}$ was 11.24 kcal/mol, or about 2 kcal/mol larger than $\Delta G^{\circ}_{\text{monomer} \rightarrow \text{dimer}}$. The dissociation constants characterizing the formation of protein dimers from monomers and of tetramers from dimers are thus 155 nM and 4.4 nM, respectively. The distribution of protein species as a function of protein concentration can be calculated from the recovered ΔG values (24). Examination of the distribution (Fig. 3 *Inset*) indicates that tetramers will comprise a significant fraction of RXRα at protein concentrations higher than 20 nM and will become the predominant species at concentrations higher than 70 nM. From these conclusions, it is expected that under the conditions employed in the nondenaturing gel electrophoresis experiment (Fig. 1), the protein will exist mainly as a tetramer. Thus, the ratio of protein in the tetramer/dimer bands is expected to be higher than that observed. This quantitative discrepancy is likely to stem from perturbation of the equilibrium distribution between the various protein species by prolonged electrophoresis. On the other hand, the fluorescence studies do not require physical separation of the species and provide information on the equilibrium distribution without perturbing it.

DISCUSSION

Analyses of the oligomeric state of RXRα in solution demonstrated that this nuclear receptor self-associated to form dimers as well as tetramers. The dissociation constants of the various oligomeric species of RXRα were estimated by examining the concentration dependence of the fluorescence anisotropy of the protein labeled with a fluorescent probe. The anisotropy changes within the titration curves were consistent with tetramer formation and indicated that tetramers formed with a pronounced cooperativity and that under the experimental conditions used, RXR tetramers became the predominant species at protein concentrations higher than 70 nM.

It is important to consider whether tetramer formation occurs at physiological protein concentrations. In regard to this point, it was reported that HL-60 human promyelocytic cells contained 1400 binding sites for all-*trans*-retinoic acid per cell and that the receptors were localized in the nucleus (29). Assuming that the radius of a nucleus is about 1 μm, we can estimate that the concentration of receptors in the nucleus will be about 550 nM. If the concentrations of RXR *in vivo* are similar to those reported for RAR in HL-60 cells, then the data presented here imply that RXR will exist predominantly as a tetramer at a physiological concentration range.

It was recently reported that the ligand-binding domain of RXRα behaved under sedimentation equilibrium conditions as a monomer and that chemical crosslinking of that domain revealed the presence of dimers but not of higher-order oligomers (30). It thus seems that tetramers are stabilized by interactive surfaces outside the ligand-binding domain, such as, for example, the dimerization interfaces in the DNA-binding domain (13–15).

Tetramer formation has not been reported for any other member of the thyroid/retinoid/retinoid receptor superfamily. Self-aggregation is, however, a recurring theme in the regulation of the action of transcription factors both in prokaryotes and in eukaryotes. Many transcription factors—e.g., retinoid receptors, the basic–helix–loop–helix–leucine zipper proteins Myc, Mad, and Max, and the protooncogene products c-Fos and c-Jun—form functionally distinct homo- and heterodimers (e.g., refs. 31–33). The *trp* repressor of *E. coli* can interact with DNA with protein/DNA ratios ranging from 2 to 8 (34, 35). Yeast heat shock transcription factor forms trimers both in solution and when bound to DNA (36), and the tumor suppressor p53 forms tetramers in solution and can form protein/DNA complexes with varying stoichiometries (37, 38).

Several hypotheses can be invoked regarding the physiological role of tetramer formation by RXR. Tetramers may function as a reservoir for the receptor, and regulation of tetramer dissociation may modulate receptor availability. In contrast with transcriptional regulation of RXR expression, controlling the availability of RXR by this mechanism would allow for rapid fine-tuning of the various hormone signaling pathways in which RXR participates. Our data indeed indicate that RXR tetramers can modulate the availability of receptor dimers for binding to cognate DNA.

Alternatively, in analogy with other regulatory DNA-binding proteins, RXR tetramers may bind to particular response elements as such. In this regard, complex response elements for retinoid receptors have been reported to exist in the promoter regions of several genes (e.g., those encoding mouse laminin B1 and rat cellular retinol-binding protein II), and the exact role of these composite response elements is not clear. It has recently been reported that binding of RXRα as well as RXR$\alpha\Delta$AB to such multiple elements involves cooperative formation of tetramers or higher-order oligomers, and it was hypothesized that the formation of these complexes is driven by the interaction with the DNA (39). In contrast, the data in the present paper demonstrate that RXR tetramers preexist in solution and, so, suggest that the observed cooperativity stems from the cooperativity in protein–protein interactions prior to DNA binding.

Tetramer formation by RXRs and factors that modulate the self-association of these proteins may constitute an additional level of regulation of cellular signaling by retinoids.

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