Individual Subunits of Heterodimers Comprised of Retinoic Acid and Retinoid X Receptors Interact with Their Ligands Independently†

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ABSTRACT: The retinoid X receptor (RXR) is a member of a family of transcription factors, known as hormone nuclear receptors, that mediate the effects of hydrophobic hormones on gene transcription. RXR, which is activated by 9-*cis*-retinoic acid (9cRA), can modulate several signaling pathways by virtue of its ability to form heterodimers with other members of the receptor family, as, for example, the retinoic acid receptor (RAR). The roles of the individual receptors within heterodimers are not clear as yet. It was recently reported that heterodimerization inhibits transcriptional activation by RXR, an effect that was attributed to an inability of RXR within heterodimers to bind its ligand. This inhibition was reported to depend on the association of heterodimers with cognate DNA and on the level of saturation of the RAR subunit within the heterodimers. In the present work, the ligand-binding characteristics of RXR and RAR individually and within heterodimers were examined by fluorescence-based methods. The results indicate that heterodimerization with RAR does not alter the ligand-binding capacity of RXR nor the rate of dissociation of the ligand from this receptor. The ligand-binding capacity of RXR also was not affected by association of heterodimers with cognate DNA nor by the level of saturation of the RAR subunit. The data indicate further that the affinity of RAR for 9cRA is considerably higher as compared to RXR and that this differential affinity is retained within RAR−RXR heterodimers. Thus, binding of ligands by subunits within RAR−RXR heterodimers proceeds independently.

The retinoid X receptor is a member of a family of nuclear receptors that serve to mediate the effects of small lipophilic hormones on gene transcription. This family also includes the retinoic acid receptors (RARs), the vitamin D receptors (VDRs), the thyroid hormone receptors (TRs), the peroxisome proliferator activated receptors (PPARs), and a number of orphan receptors (Giguère, 1994; Chambon, 1994). Among the hormone nuclear receptors, RXR displays unusual protein−protein interaction properties. According to current knowledge regarding the self-association of members of the receptor family, RXR is the only protein that exists in solution as a tetramer (Kersten *et al*., 1995a). This oligomerization state of RXR is regulated by its ligand 9-*cis*-retinoic acid (9cRA) (Kersten *et al*., 1995b). RXR also stands out by virtue of its ability to interact with other members of the receptor family to form heterodimers (Giguère, 1994; Chambon, 1994). Heterodimerization of RXR with RAR, VDR, TR, etc. enhances the binding affinity of these receptors for cognate DNA and can alter the specificity of the interactions of the receptors with response elements (Glass, 1994). Heterodimer formation by RXR thus allows this receptor to function as a key regulator for signals from various converging hormonal pathways.

The functional role of each monomer within RXR-containing heterodimers is not clear at present. Potentially, heterodimerization of RAR and RXR can result in the convergence of signaling by two different retinoids: *all-trans*-retinoic acid (tRA), which binds to RAR, and 9cRA, which binds to both receptors (Levin *et al*., 1992; Heyman *et al*., 1992; Allenby *et al*., 1993; Allegretto *et al*., 1993). A number of studies indeed showed that in cells in which RXR and RAR are coexpressed, 9cRA is more effective in inducing transactivation than tRA regardless of the type of reporter construct used (Durand *et al*., 1992; Zhang *et al*., 1992; Heery *et al*., 1993; Lee *et al*., 1995; Chen *et al*., 1995). As 9cRA interacts with both receptors while tRA binds exclusively to RAR, these observations suggest that ligand binding by both the RXR and RAR subunits within the heterodimer is required for high levels of transactivation. It was also reported that activation by RAR- and RXR-selective ligands occurs independently and is additive (Durand *et al*., 1994), and that efficient inhibition of activation-induced T-cell apoptosis requires the occupancy of both RXR and RAR by ligands, indicating that RAR−RXR heterodimers are activated by ligands for both receptors (Yang *et al*., 1995).

In contrast, it was reported that when cells are transfected with expression vectors for RAR and RXR, ligands that specifically bind to RXR are ineffective in enhancing transactivation (Lehmann *et al*., 1992; Kurokawa *et al*., 1994; Valcárel *et al*., 1994; Forman *et al*., 1995a). Kurokawa *et al*. (1994) further studied possible mechanisms for the inefficiency of the RXR-specific ligand in transactivation *via* RAR−RXR heterodimers by examining the interactions of the RXR-selective ligand LG1069 and of 9cRA with RAR−RXR heterodimers *in vitro*. These investigators concluded that, within a heterodimer, RAR allosterically inhibits ligand binding by RXR. This inhibition could be observed only

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when the heterodimer was associated with cognate DNA. More recently, the same issue was examined by Forman *et al*. (1995a), who proposed a refinement of the original allosteric model. It was suggested in the latter study that RAR inhibits ligand binding by RXR within a RAR−RXR heterodimer only when RAR is unliganded. Once RAR is liganded, the inhibition is released, and RXR is able to bind ligand (Forman *et al*., 1995a).

The present work was undertaken to examine the effects of formation of RAR−RXR heterodimers on the ligand-binding characteristics of RXR. The data reveal that heterodimerization with RAR does not alter the ligand-binding capacity of RXR nor the rate of dissociation of ligand from this receptor. It is also shown that neither the association of RAR−RXR heterodimers with cognate DNA nor the level of saturation with ligand of RAR within the heterodimer affects the ligand-binding affinity of the RXR subunit. The data further indicate that the affinity of RAR for 9cRA is considerably higher as compared to RXR and that this differential affinity is retained within RAR−RXR heterodimers. Taken together, these data strongly suggest that binding of ligands to the individual subunits within a RAR−RXR heterodimer proceeds independently.

MATERIALS AND METHODS

Ligands. 9cRA and LG1069 were gifts from Hoffmann-LaRoche (Nutley, NJ) and Ligand Pharmaceuticals (San Diego, CA), respectively. tRA was purchased from Kodak. SR11246 was synthesized as described (Dawson *et al*., 1995).

Antibodies. Monoclonal antibodies for RXR and RAR were provided by Hinrich Gronemeyer and Pierre Chambon.

Proteins. In this study, RXRα and RARα lacking the N-terminal A/B domain (RXRαΔAB, RARαΔAB) were used. The text of this paper refers to these truncated proteins as RXR and RAR. Proteins were obtained by overexpression in *Escherichia coli* essentially as described (Chen *et al*., 1994). *E. coli* harboring the RXRαΔAB or the RARαΔAB gene on the pET15b plasmid was grown to 0.8−1.2 ODU at 37 °C. Following induction with 0.5 mM IPTG, cells were grown for an additional 2.5 h. Cell lysis and protein purification were followed as described (Chen *et al*., 1994) except for the omission of freeze−thaw cycling in cell lysis. Representative Coomassie blue-stained SDS−PAGE gels of isolated RAR and RXR are shown in Figure 1. Isolated receptors from numerous preparations were usually found to possess 0.6−0.85 mol of ligand-binding sites per mole of protein. As shown in Chen *et al*. (1994), in Kersten *et al*. (1995a−c), and in the present work, these proteins show the expected ligand-selectivity: RAR binds both tRA and 9cRA and does not interact with RXR-selective ligands, while RXR binds only 9cRA and several RXR-selective ligands. In addition, in agreement with previous reports, the affinity of binding of 9cRA to RAR is significantly stronger than the binding of this ligand to RXR. The receptors also display the expected specificity for consensus response elements; for example, RXR binds with a significantly stronger affinity to a DR1 *vs* to a DR5 response element, while RAR preferentially associates with a DR5 RE. Protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

Covalent labeling of RXR with the fluorescent probe fluorescein was carried out as described previously (Kersten *et al*., 1995a).

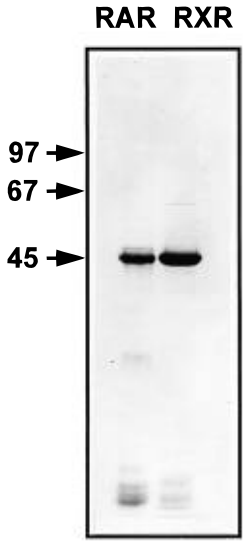

FIGURE 1: SDS−PAGE gels of isolated RAR and RXR. RAR and RXR overexpressed in *E. coli* were purified as is detailed under Materials and Methods. Purified proteins were electrophoresed on a SDS−PAGE gel and stained by Coomassie blue.

Oligonucleotides. Oligonucleotides containing the response elements DR5 or DR1 were synthesized and purified at the Cornell Biotechnology Center. Single-stranded DNA was annealed (Kadonaga & Tijan, 1986) and double-stranded DNA isolated on Centrex centrifugal filter units (Schleicher & Schuell).

DR5

5′-AGCTTGGCGCCGGGTCACCGAAAGGTCAG-3′

DR1

5′-TCGAGGGTAGGGGTCAGAGGTCACTCG-3

Unilamellar Vesicles. Small unilamellar vesicles of dioleoylphosphatidylcholine (DOPC) were prepared by sonication. Chloroform used to dissolve the lipid was evaporated under argon and further removed by drying under vacuum for 2 h. Lipids were suspended in buffer A (10 mM Hepes, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 400 mM KCl, and 5% glycerol) containing 100 mM KCl. The suspension was sonicated to clarity using a Heat-System sonicator, and centrifuged at 100000*g* for 10 min to pellet multilamellar vesicles. Lipid concentration was determined based on phosphorus content according to Dittmer and Wells (1969).

Fluorescence Titrations. Retinoid receptors were mixed in buffer A containing 100 mM KCl. For ligand-binding studies with RXR or RAR alone, receptors were used at a concentration of 1 μM. For binding studies with heterodimers of RAR−RXR, concentrations of either 0.75 or 1 μM of each receptor were used. In some experiments, an oligonucleotide containing a DR5 response element was added in 3-fold molar excess over protein. Receptor mixtures were titrated with 9cRA or tRA in concentrated ethanol solutions or with SR11246 dissolved in dimethyl sulfoxide (DMSO). Binding of ligand was monitored by following the changes in the intrinsic fluorescence of the proteins (λ_{ex} = 280 nm; λ_{em} = 340 nm). Ligand binding to RXR within homo- and heterodimers was also monitored by using fluorescein-labeled receptor. The labeled receptor was titrated with 9cRA, or tRA, or with the RXR-specific ligand

SR11246, and the fluorescence of the probe (λ_{ex} = 491 nm; λ_{em} = 516 nm) was monitored.

Titration curves were corrected for inner-filtering reflected by a linear nonspecific decrease in fluorescence following saturation (Cogan *et al*., 1976). Corrected data were analyzed to yield the dissociation constant (K_d) and the number of ligand-binding sites. Analyses were carried out both by the linearization method describd by Cogan *et al*. (1976) and by using an equation derived from simple binding theory (Norris *et al*., 1994). Nonlinear least-squares regressions were carried out using the software Origin (MicroCal, Inc.).

Kinetics of Transfer of 9cRA between Receptor Oligomers and Unilamellar Vesicles of DOPC. Dissociation of 9cRA from homo- and heterodimers of RXR and RAR was initiated by mixing the protein(s) with vesicles of DOPC using a stopped-flow accessory. Proteins (1 μM), complexed with the ligand, were mixed at a volume ratio of 1:1 with vesicles of DOPC (2 mM lipid), both in buffer A containing 100 mM KCl. Movement of the ligand from the protein to the vesicles was followed by monitoring the time-dependent increase in the intrinsic fluorescence of the protein (λ_{ex} = 280 nm; λ_{em} = 340 nm). Alternatively, RXR labeled with the fluorescent probe fluorescein was used, and movement of ligand between protein and vesicles was followed by monitoring the time-dependent increase in the fluorescence of the probe (λ_{ex} = 491 nm; λ_{em} = 516 nm).

Competition between SR11246 and 9cRA on Binding to RXR. RXR alone or mixed with RAR (1 μM each) was complexed with 9cRA in buffer A containing 100 mM KCl. The receptor(s)−ligand complex was titrated with SR11246 from a concentrated solution in DMSO, and the increase in the intrinsic fluorescence of the protein complex (λ_{ex} = 280 nm; λ_{em} = 340 nm) was measured. For studies with DNA-bound heterodimers, a 3-fold molar excess of DR5 was added to the sample mixture.

Electrophoretic Mobility Shift Assays (EMSA). RXR and RAR (1 μM each) were mixed in the presence of a 3-fold molar excess of DR5 in buffer A containing 100 mM KCl. Protein-DNA complexes were resolved by nondenaturing gel electrophoresis on a 5% polyacrylamide gel (0.5× TBE; 1.5 h prerun at 100 V; 2−2.5 h run at 30 mA). During electrophoresis, the gel was cooled with circulating tap water.

Western Blots. Protein−DNA complexes were electrophoresed as described above for EMSAs. Protein bands were transferred to a PVDF membrane (24 V; 3−4 h; 4 °C). The membrane was incubated overnight at 4 °C in Tris-buffered saline containing 0.1% Tween and 5% dry milk to block nonspecific binding. After extensive washing with TBS−Tween, the membrane was cut and the two parts incubated separately with monoclonal antibodies against either RXR or RAR (1:500 dilution) for 1 h at room temperature. Membranes were washed, incubated with anti-mouse IgG antibody labeled with horseradish peroxidase (1:500 dilution) for 1 h, and developed according to ECL Western Blotting protocols (Amersham).

RESULTS

Ligand-Binding Capacity of RXR Is Not Affected by Heterodimer Formation. Due to the extensive overlap of the absorption spectrum of retinoids with the fluorescence emission spectra of tryptophans and tyrosines, binding of retinoids to proteins is often accompanied by quenching of the intrinsic fluorescence of the protein. This phenomenon has been widely used to study ligand binding by a variety of retinoid-binding proteins including RXR (e.g., Cogan *et al*., 1976; Ong & Chytil, 1980; Chen & Noy, 1994; Chen *et al*., 1994). This fluorescence-based methodology does not require physical separation of free from bound ligand and is especially useful in studying retinoids since these ligands are hydrophobic and have a tendency to adhere to matrixes used to separate different species in binding assays utilizing radioactive ligands. However, due to the limits of the sensitivity of spectrofluorometers and the lower stability of proteins in very dilute solutions, these measurements usually are carried out using protein concentrations that are higher than 100 nM. These concentrations are significantly higher than the reported dissociation constants (K_d) of the complexes of retinoic acids with either RXR or RAR, and under these conditions, titration curves reflect stoichiometric rather than equilibrium binding. The extracted K_d values thus report upper limits rather than precise estimates. Nevertheless, fluorescence titrations do accurately report on the stoichiometry of ligand binding regardless of the concentration or the binding affinity of the protein studied.

Fluorescence titrations of RXR and RAR with 9cRA are shown in Figure 2. Comparison of data for RXR (Figure 2A) and for RAR (Figure 2B) shows that both the intensity and the change in fluorescence upon ligand binding are more pronounced for the former. These observations most likely reflect the presence of two tryptophans per RXR molecule *vs* one tryptophan in RAR, and possibly a different distance or orientation between the tryptophans and the ligand within the binding sites of the two proteins. The titration curves were corrected for inner-filtering by the ligand which gave rise to a shallow, linear decrease in fluorescence observed following saturation of the proteins (Cogan *et al*., 1976). Corrected curves were analyzed to obtain the number of binding sites and the K_ds of protein−ligand complexes. Analyses were carried out both by the linearization method described by Cogan *et al*. (Figure 2, insets) and by fitting the data to an equation based on simple binding theory (solid lines through data points in Figure 2). In all cases, values for the number of binding sites obtained from curve-fitting and from the linearization method agreed within 5%. The values for K_ds varied more widely but were all within the several nanomolar to 25 nM range. The values extracted by curve-fitting are shown in Table 1. These data verify that both RAR and RXR were about 70% functionally active and bound 9cRA with high affinity. Titrations of RAR−RXR heterodimers with 9cRA (Figure 2C) and analyses of the data (Table 1) revealed that the heterodimers possessed the full complement of binding sites for 9cRA.

Fluorescence titrations of the receptors with tRA were also carried out. As previously reported, tRA associated with RXR very weakly (Allenby *et al*., 1993) but bound to RAR with high affinity. As expected, RAR was found to possess the same number of binding sites for tRA and 9cRA, while the RAR−RXR heterodimer exhibited only half of the number of binding sites for tRA as those found for 9cRA (Table 1). These observations reflect that only the RAR partner within the heterodimer bound this ligand and thus that the ligand selectivities of the two receptors were retained upon heterodimerization.

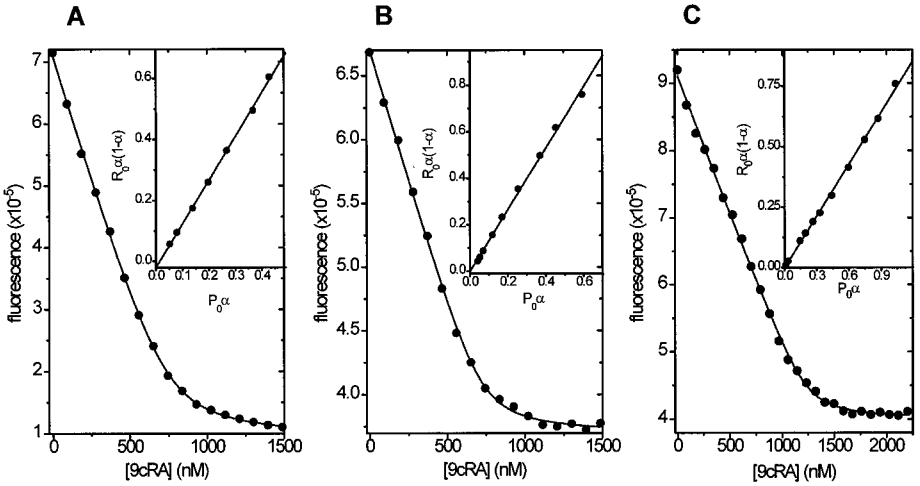

FIGURE 2: Fluorescence titrations of RXR and RAR homo- and heterodimers with 9cRA. RXR (A), RAR (B), or RAR−RXR (C), each at 1 μM concentration, were titrated with 9cRA from a concentrated solution in ethanol, and binding of ligand to the receptor was monitored by following the intrinsic protein fluorescence (λ_{ex} = 280 nm; λ_{em} = 340 nm). Titration curves were corrected for inner-filtering effects (Cogan *et al*., 1976). The solid line through the data points represents the fit of the data according to eq 1 (see Materials and Methods). Insets show analyses of the data by the linerization method described by Cogan *et al*. (1976).

Table 1: Number of Binding Sites (#) and Upper Limits for Dissociation Constants (K_d) Characterizing the Association of RAR, RXR, and RAR−RXR Heterodimers with Ligands[a]

			ligands					
			tRA		9cRA		SR11246	
receptor	DNA	other ligands	#	K_d	#	K_d	#	K_d
RAR	−	−	0.69*	20*	0.67 ± 0.04	16 ± 4		
RXR	−	−			0.74 ± 0.03	20 ± 3	0.71 ± 0.06	22 ± 8
RAR−RXR	−	−	0.72**	33**	1.32 ± 0.04	9 ± 2		
RAR−RXR	+	−			1.37 ± 0.05	2 ± 1	0.61 ± 0.01	12 ± 5
RAR−RXR	+	tRA					0.71 ± 0.02	24 ± 9

[a] Data were obtained by fluorescence titrations as described in the text. Receptors (1 μM each) were titrated with the denoted ligands. Ligand binding was followed by monitoring the decrease of the intrinsic fluorescence of the proteins (titrations with tRA and with 9cRA) or by following the fluorescence of fluorescein covalently bound to RXR (titrations with SR11246). Data analyses were carried out as described in the legend to Figure 1. K_ds are expressed in nanomolar. # is the micromolar concentration of ligand bound at saturation. n = 3 except values marked by an asterisk where n = 2 or with two asterisks where n = 1.

Ligand-Binding Capacity of RXR within RAR−RXR Heterodimers Is Not Affected by Association of the Heterodimer with Cognate DNA. As discussed in the introduction, it has been reported that RXR within a RAR−RXR heterodimer is unable to bind its ligand when the heterodimer is associated with cognate DNA (Kurokawa *et al*., 1994). To examine the effect of association with cognate DNA on the ligand-binding properties of a RAR−RXR heterodimer, the interactions of 9cRA with DNA-bound heterodimers were studied. Oligonucleotides containing either a DR5 or a DR1 response element were used in a 3-fold molar excess over heterodimer. Under these conditions, RAR and RXR were present as a DNA-bound heterodimer as was verified by electrophoretic mobility-shift assays followed by Western blotting with monoclonal antibodies against either RXR or RAR. Figure 3 shows a Western blot of an EMSA of a mixture of equimolar concentrations of RAR and RXR, and a 3-fold molar excess of oligonucleotides containing a DR5 response element. One major protein band could be detected by using antibodies against either RAR or RXR. This band coincided with a band observed by autoradiography of gels on which mixtures of receptors and ^{32}P-labeled oligonucleotides were resolved, confirming that the receptor was present mainly as a DNA-bound heterodimer. A minor band was observed by blotting with antibodies for RXR. As this band displayed a higher mobility, it most likely reflects a small amount of degraded DNA-bound RXR.

Fluorescence titrations with 9cRA revealed that the DNA-bound RAR−RXR heterodimer possessed the full complement of binding sites for this ligand (Table 1). The data thus showed that binding of a RAR−RXR heterodimer to DNA does not hinder the ability of RXR within the complex to interact with its ligand.

Differential Affinities of RAR and RXR for 9cRA Are Retained within Heterodimers and Are Not Influenced by Binding to Cognate DNA. 9cRA can bind to and activate both RAR and RXR, but it has been reported that this ligand associates with RAR with a significantly higher affinity as compared to RXR (Allegretto *et al*., 1993; Allenby *et al*., 1993). Considering the importance of heterodimerization in retinoid signaling, and taking into account that the interactions of heterodimers with cognate DNA are usually tighter as compared with those of the individual receptors, it was of interest to examine whether the differential affinity of the two proteins for 9cRA is retained upon heterodimer formation.

To distinguish between binding of 9cRA to RXR and to RAR within RAR−RXR heterodimers, RXR was covalently labeled with the fluorescent probe fluorescein. Labeling did not affect the ligand-binding or the DNA-binding properties

FIGURE 3: Western blot of an EMSA of RAR−RXR heterodimers in the presence of a DR5 response element. RAR and RXR (1 μM each) were incubated with a 3-fold molar excess of DR5 oligonucleotide in a 30 μL reaction volume. Sample mixtures were resolved by nondenaturing gel electrophoresis. Protein−DNA complexes were transferred to a PVDF membrane, followed by Western blotting using anti-RXR and anti-RAR antibodies (see Materials and Methods for experimental details).

of the receptor, as was verified by fluorescence titrations with 9cRA and by electrophoretic mobility shift assays (data not shown). Labeling also did not affect the ability of RXR to self-associate to dimers and to tetramers (Kersten *et al*., 1995a−c). The fluorescence of fluorescein-labeled RXR was previously found to decrease in a saturable fashion upon titration of the labeled receptor with 9cRA (Kersten *et al*., 1995c) and can be used as an efficient readout for monitoring ligand binding by the protein.

A representative titration of heterodimers comprised of RAR and fluorescein-labeled RXR with 9cRA is shown in Figure 4. In this experiment, in which the fluorescence of the labeled RXR was followed, the observed changes solely reflected effects on the RXR moiety of the heterodimer. An initial linear and shallow phase and a second phase characterized by a steep decrease in fluorescence and followed by a plateau were observed. The initial phase reflected changes in RXR that were induced by binding of ligand to the RAR moiety. This could be inferred from the observation that a similar pattern emerged upon titration of the heterodimer with tRA which does not bind to RXR (Figure 4, inset). The second phase reflected binding of 9cRA by RXR and was followed by a plateau which was reached at saturation. These data indicated that RAR within the heterodimer approached saturation with 9cRA prior to binding of the ligand to the RXR partner. Such a pattern of ligand binding in which the first ligand-binding site approaches saturation before appreciable ligand binding in the second site occurs is characteristic of the existence of independent binding sites with significantly different binding affinities. The data thus

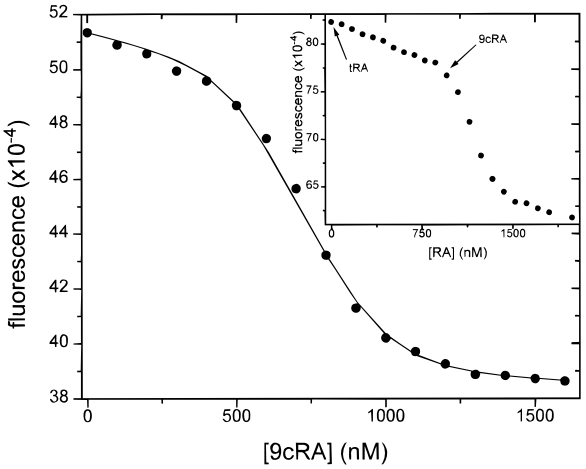

FIGURE 4: Fluorescence titration of heterodimers of RAR and fluorescein-labeled RXR with 9cRA. Fluorescein-labeled RXR and unlabeled RAR (1 μM each) were titrated with 9cRA. Binding of ligand to RXR within the heterodimer was followed by monitoring the fluorescence of the labeled RXR (λ_{ex} = 491 nm; λ_{em} = 516 nm). The solid line through the data points represents the fit of the data to a binding isotherm assuming two independent binding sites. Fits were carried out using a computer program which allows for analysis of multiple equilibria (Royer *et al*., 1990). Preparations of receptors used in this experiment contained only 0.5 mol of ligand-binding sites per mole of protein. Data fitting was carried out using this value. The inset to the figure shows a titration of the heterodimers with tRA. Subsequent to saturation of the RAR moiety with tRA, the heterodimer was further titrated with 9cRA. These data demonstrate that ligand binding by RAR within the heterodimer somewhat affects the fluorescence of the labeled RXR within the complex (see text).

indicate that the ligand-binding affinity of RAR within the heterodimer was considerably higher as compared to that of the RXR partner.

The data in Figure 4 could be well fitted according to the above model using the BIOEQS computer program that allows for analysis of multiple equilibrium (Royer *et al*., 1990; solid line in Figure 4). Analyses of the data yielded a K_d for binding of 9cRA to RAR and to RXR within the heterodimer of 0.13 nM and 16 nM, respectively (mean of two experiments). These values agree remarkably well with published estimates for the equilibrium dissociation constants for binding of 9cRA to RAR and RXR individually (Levin *et al*., 1992; Heyman *et al*., 1992; Allenby *et al*., 1993; Allegretto *et al*., 1993). The data thus show that heterodimerization does not affect the affinity of either RAR or RXR for 9cRA.

To further examine the effect of association with cognate DNA on the interactions of RAR−RXR heterodimers with ligands, the binding affinities for 9cRA within heterodimers composed of RAR and fluorescein-labeled RXR were measured in the presence of cognate DNA. Following verification that under the conditions used the protein was bound to DNA as heterodimers (Figure 3), the receptors were titrated with 9cRA, and the fluorescence of the RXR-bound fluorescein was monitored. The titration curves were very similar to the curve shown in Figure 4 which was obtained in the absence of a DNA template. Analyses of the titration curves yielded K_ds for binding of 9cRA to RAR and to RXR within the DNA-bound heterodimer of 0.15 and 11 nM, respectively. The similarity of the K_d values for the interactions of 9cRA with RAR and RXR within heterodimers, in solution and when bound to cognate DNA,

provides further evidence that association of a RAR−RXR heterodimer with DNA does not affect the ligand-binding properties of the receptors.

Heterodimerization Does Not Affect the Rate of Dissociation of 9cRA from RXR or from RAR. The data suggested that heterodimerization does not alter the binding affinities of either RAR or RXR for 9cRA. To further examine the effects of formation of RAR−RXR heterodimers on the ligand-binding characteristics of the receptors, the rate constants for dissociation of 9cRA from the receptors, individually and within a heterodimer, were measured.

The equilibrium dissociation constant of a protein−ligand complex relates to the rate constant of dissociation of the complex (k_{off}) and the rate constant for association of the two components (k_{on}) according to $K_d = k_{off}/k_{on}$. Changes in binding affinity between receptor and ligand can thus stem from changes in k_{off}, in k_{on}, or in both parameters. The rates of formation of the complexes of retinoid receptors with their ligands are too rapid to allow for measurements by the methodologies used here. However, it can be expected that significant variations in binding affinities will also result in changes in k_{off}, which is a measurable parameter. To induce dissociation of the ligand from the receptors, protein was complexed with 9cRA and mixed with unilamellar vesicles of dioleoylphosphatidylcholine (DOPC). Due to the high affinity of lipid bilayers for retinoic acid (Noy, 1992), following the introduction of lipid vesicles into solution containing receptor−ligand complexes, the ligand moves from the receptor to the vesicles. Since the rate constant of dissociation of the receptor−ligand complex is the slowest step in the transfer process, the rate constant for movement of 9cRA from the proteins to the vesicles directly reflects the rate constant of dissociation from the donor protein [see Doody *et al*. (1980), Noy and Xu (1993), and Noy and Blaner (1991)].

Binding of 9cRA to RAR or RXR results in quenching of the intrinsic fluorescence of the protein. Movement of 9cRA from receptor to vesicles could thus be followed by monitoring the time-dependent release of quenching of protein fluorescence. To monitor transfer of 9cRA from RAR alone to vesicles, ligand was complexed with RAR at a molar ratio of 0.5. To monitor transfer of 9cRA from RAR that is part of a RAR−RXR heterodimer, the ligand was complexed with the heterodimer at a molar ratio of 0.3, a saturation level at which all of the 9cRA within the heterodimer is bound to the RAR moiety (see Figure 4). Representative traces of the time-dependent movement of 9cRA from RAR alone and from RAR within a RAR−RXR heterodimer to lipid vesicles are shown in Figure 5. The data could be well described as a single first-order reaction (solid line in Figure 5), yielding rate constants of 0.041 ± 0.003 s^{-1} and 0.051 ± 0.005 s^{-1} (mean ± SEM; $n = 3$ or 5) for dissociation of 9cRA from RAR alone and from RAR within the heterodimer, respectively, corresponding to $t_{1/2}$s of 16.9 and 13.5 s. These data indicate that heterodimerization does not significantly influence the rate of dissociation of 9cRA from RAR.

The rate constant for dissociation of 9cRA from homodimeric RXR was previously measured and was found to be 0.14 s^{-1}, corresponding to a $t_{1/2}$ of 4.95 s (Kersten *et al*., 1995c). Hence, the rate constant for dissociation of 9cRA from RAR is about 3-fold slower as compared with RXR, in agreement with the higher binding affinity of the former receptor for this ligand. To monitor the dissociation of 9cRA from RXR within a RAR−RXR heterodimer, fluorescein-labeled RXR was used. Ligand binding by labeled RXR is accompanied by a decrease in the fluorescence of the RXR-bound probe. Movement of ligand from RXR to lipid vesicles thus results in an increase in probe fluorescence. To measure the rate of movement of 9cRA from the RXR moiety of a RAR−RXR heterodimer, a ligand/heterodimer molar ratio of 1 was used. Under these conditions, a significant fraction of 9cRA is bound to the RXR moiety (see Figure 4).

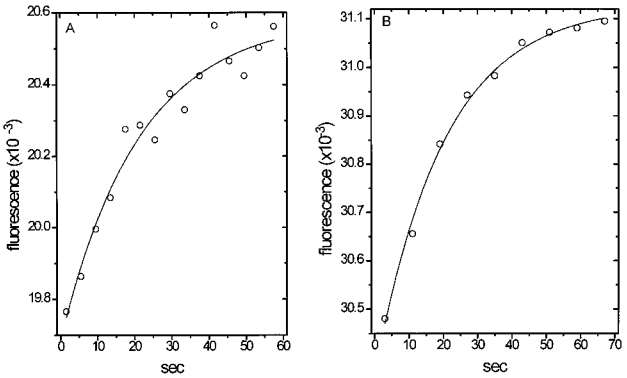

FIGURE 5: Transfer of 9cRA from RAR to unilamellar vesicles of phosphatidylcholine. 9cRA was complexed with RAR (1 μM) at a ligand/protein molar ratio of 0.5 (A) or with a RAR−RXR heterodimer (1 μM) at a molar ratio of 0.3 (B). Complexes were mixed with small unilamellar vesicles of DOPC (2 mM lipids) using a stopped-flow accessory. The intrinsic fluorescence of the receptor(s) (λ_{ex} = 280 nm; λ_{em} = 340 nm) was followed until equilibrium was reached. Lines represent the fit of the data according to a single first-order reaction.

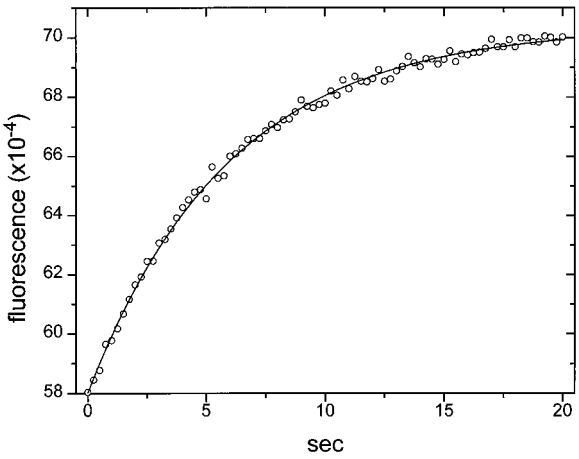

FIGURE 6: Transfer of 9cRA from a heterodimer of RAR with fluorescein-labeled RXR to unilamellar vesicles of phosphatidylcholine. 9cRA was complexed with a heterodimer of RAR and fluorescein-labeled RXR (1 μM each) at a ligand/complex molar ratio of 1. The complex was mixed with small unilamellar vesicles of DOPC (2 mM) using a stopped-flow accessory. The fluorescence of fluorescein-labeled RXR (λ_{ex} = 491 nm; λ_{em} = 516 nm) was followed until equilibrium was reached. Lines represent the fit of the data according to a single first-order reaction.

A representative trace of the time-dependent increase in fluorescence of fluorescein-labeled RXR upon mixing of liganded RAR−RXR heterodimer with vesicles is shown in Figure 6. The transfer reaction could be well described as a single first-order reaction and yielded a k_{off} for dissociation of 9cRA from RXR within a RAR−RXR heterodimer of 0.17 ± 0.001 s^{-1} (mean ± SEM, $n = 4$) corresponding to a $t_{1/2}$ of 4.1 s. This value is very similar to the k_{off} for the dissociation of 9cRA from homodimeric RXR, suggesting

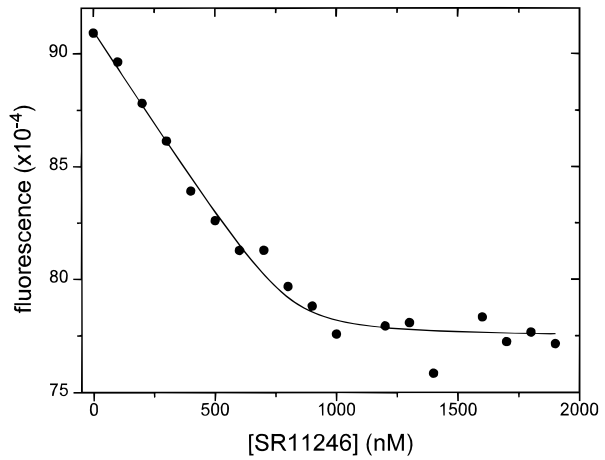

FIGURE 7: Fluorescence titration of fluorescein-labeled RXR with SR11246. Fluorescein-labeled RXR (1 μM) was titrated with SR11246 from a concentrated solution in DMSO. Ligand binding was followed by monitoring the fluorescence of the fluorescein-labeled RXR (λ_{ex} = 491 nm; λ_{em} = 516 nm). Titration curves were corrected for the nonspecific linear decrease in fluorescence observed upon receptor saturation with ligand and analyzed as detailed in the legend to Figure 2.

that heterodimerization does not significantly influence the rate of dissociation of RXR−ligand complexes.

Level of Saturation of RAR within a Heterodimer Does Not Affect Ligand Binding by the RXR Moiety. The data presented above, taken together, demonstrate that formation of RAR−RXR heterodimers does not lead to significant alterations in the ligand-binding characteristics of either of the partner receptors. It has been suggested, however, that heterodimerization hinders the interactions of the RXR moiety with its ligand, and that binding of ligand to RAR within the heterodimer leads to release of this inhibition (Forman *et al*., 1995a). The data in Figure 4 indicate that, within a heterodimer, the differential affinity of the two receptors for 9cRA results in saturation of the RAR subunit prior to ligand binding by RXR. Consequently, use of 9cRA does not provide information regarding the ability of heterodimeric RXR to bind ligand in the presence of unliganded RAR. Examination of this model thus requires the use of a RXR-selective ligand which will allow for studying ligand binding by heterodimeric RXR without presaturating the RAR partner.

To address this issue, the synthetic compound SR11246, which was shown to be highly selective for RXRα (Dawson *et al*., 1995), was used. SR11246 does not absorb light at wavelengths longer than 300 nm, and its addition to retinoid receptors does not result in measurable quenching of the intrinsic fluorescence of the proteins. To study the binding of SR11246 to RXR, fluorescein-labeled receptor was again used. Similar to the observation with 9cRA, titration of fluorescein-labeled RXR with SR11246 was accompanied by a saturable decrease in the fluorescence of the probe, and the change in fluorescence could be used to monitor binding of this ligand to RXR (Figure 7). Analysis of the titration curve demonstrated high affinity binding of this ligand to RXR (Table 1).

It was previously shown that RXR exists in solution as a tetramer and that binding of 9cRA to the receptor leads to rapid dissociation of tetramers to dimers (Kersten *et al*., 1995a−c). It was of interest therefore to examine the effect of binding of SR11246 on the oligomeric state of RXR. Nondenaturing gel electrophoresis of RXR in the presence and absence of SR11246 indicated that, similarly to 9cRA, SR11246 induced dissociation of RXR tetramers (data not shown).

To investigate whether SR11246 is able to bind to RXR that is part of a DNA-bound RAR−RXR heterodimer, a mixture of RAR, fluorescein-labeled RXR, and an oligonucleotide containing the DR5 response element was titrated with this ligand. Analyses of the titration curves (Table 1) demonstrated that the RXR-selective ligand bound to the full complement of RXR within the DNA-bound heterodimer under conditions in which the RAR moiety was unliganded. A similar result was obtained in the presence of a saturating concentration of tRA (Table 1), further demonstrating that the level of saturation of RAR had no effect on the binding properties of RXR within RAR−RXR heterodimers.

To verify that data obtained utilizing SR11246 do not report observations that are unique to this ligand, titrations were carried out using another RXR-selective ligand, LG1069. This ligand, similarly to SR11246, bound with a high affinity to the full complement of RXR within the DNA-bound heterodimer in the absence as well as in the presence of a saturating concentration of tRA.

In agreement with the conclusions of the present study, it has been reported that RAR and RXR within heterodimers were activated independently by their ligands when reporters containing either the DR1 or the DR5 response elements were used (Durand *et al*., 1994). However, it was noted in that study that when a DR5T response element was used (AGTTCAnnnnnAGTTCA), no activation by an RXR-selective ligand could be observed. Fluorescence titrations of heterodimers bound to the DR5T response element were carried out in order to clarify whether RXR within heterodimers loses its ligand-binding capability when bound to this response element. No such inhibition could be observed (data not shown).

SR11246 Has a Lower Affinity for RXR As Compared with 9cRA. To better estimate the relative affinities of SR11246 and 9cRA for RXR, fluorescence competition assays were carried out. In contrast to binding of 9cRA to RXR, binding of SR11246 does not cause quenching of the intrinsic fluorescence of the protein. Replacement of RXR-bound 9cRA by SR11246 will thus result in release of quenching of the protein fluorescence caused by 9cRA and will be accompanied by an increase in fluorescence. RXR complexed with 9cRA (0.7 mol of 9cRA per mole of protein) was titrated with SR11246 (Figure 8). The data showed a saturable increase in protein fluorescence, indicating that 9cRA was effectively competed off the receptor as the concentration of SR11246 was increased. The EC_{50}, the concentration of SR11246 at which half the 9cRA was displaced from RXR, was found to be 1.75 μM, indicating that the affinity of 9cRA for the receptor was 2.5-fold higher than that of SR11246. Similar experiments conducted using a RAR−RXR heterodimer in solution or a DNA-bound heterodimer yielded EC_{50} values of 2.2 and 2.6 μM, respectively (data not shown). These observations indicate that the affinity of SR11246 for RXR is about 3-fold lower than the affinity of 9cRA for this receptor.

DISCUSSION

Heterodimerization of transcription factors allows for integration of signals from various hormonal pathways, and

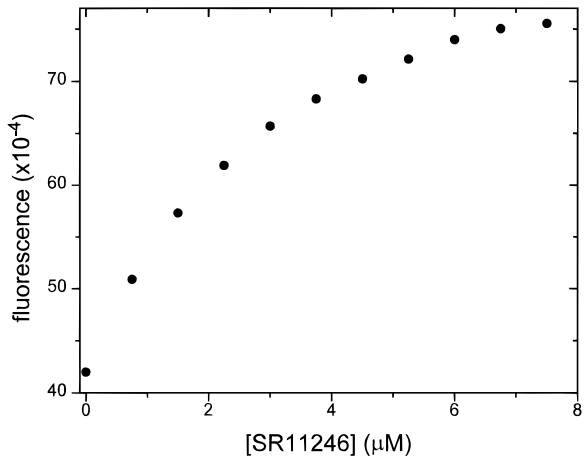

FIGURE 8: Competition between SR11246 and 9cRA on binding to RXR. RXR (1 μM) was complexed with 9cRA (0.7 μM) and titrated with SR11246 from a concentrated solution of DMSO. The release of quenching of the intrinsic fluorescence of the protein complex (λ_{ex} = 280 nm; λ_{em} = 340 nm) reflected the exchange of protein-bound 9cRA with SR11246 as the concentration of the later ligand was increased.

can greatly increase the complexity of the transcription modulation process. Within the family of nuclear hormone receptors, heterodimerization with RXR interconnects signaling by the retinoic acid receptor, the thyroid hormone receptor, the vitamin D receptor, and the peroxisome proliferator activated receptor. A number of new members of this family were recently shown to be able to dimerize with RXR (Perlmann & Jansson, 1995; Willy *et al*., 1995; Forman *et al*., 1995b), further underlining the pivotal role of RXR in coupling numerous signaling pathways.

Formation of heterodimers with RXR enhances the binding affinity and alters the specificity of the interactions of the individual receptors with cognate DNA (Glass, 1994). However, the roles of the individual subunits within RXR-containing heterodimers remain elusive. Heterodimers of RXR with RAR can potentially respond to two different retinoids which selectively bind to the individual dimer subunits. (Durand *et al*., 1992, 1994; Zhang *et al*., 1992; Heery *et al*., 1994; Lee *et al*., 1995; Chen *et al*., 1995; Yang *et al*., 1995). However, it has also been reported that the ligand-dependent transactivation function of RXR is silent within RAR−RXR heterodimers (Kurokawa *et al*., 1994; Valcárel *et al*., 1994; Forman *et al*., 1995a). The loss of responsiveness to a RXR-selective ligand may stem from the loss of the ligand-induced transcriptional activation function within the heterodimer, or, alternatively, the inhibition may originate from loss of the ability of the RXR moiety within the heterodimer to bind its ligand. Based on the results of competition binding studies using the radioactive ligands LG1069 and 9cRA, Kurokawa *et al*. (1994) concluded that within a RAR−RXR heterodimer, RAR allosterically blocks ligand binding by RXR, and that this inhibition depends on the association of the heterodimer with cognate DNA. This model was further modified by Forman *et al*. (1995a), who suggested that the allosteric inhibition of ligand binding to RXR only occurs when RAR is unliganded.

In this work, the ligand-binding characteristics of RAR and RXR alone or as parts of a heterodimer were thoroughly examined. To do so, we measured the ligand-binding stoichiometries and affinities of the receptors, and the rates of dissociation of receptor−ligand complexes. All of the measured parameters were found to be almost identical in RAR−RXR heterodimers as compared to the individual receptors, indicating that the RAR and RXR subunits within RAR−RXR heterodimers bind 9cRA independently and with high, though differential, affinity. In addition, we examined the effect of binding to cognate DNA on receptor−ligand interactions, and used RXR-selective ligands to study whether the level of saturation of RAR within a heterodimer modulates the ability of the RXR partner to interact with its ligand. The data indicate that RXR within a DNA-bound RAR−RXR heterodimer tightly associates with its ligand, and that the binding affinity is independent of the saturation level of the RAR subunit. Overall, the ligand-binding characteristics of RXR were not altered by heterodimerization, nor by association of the heterodimer with cognate DNA, nor by the level of saturation of the RAR subunit of the heterodimers. The data thus clearly demonstrate that binding of ligands to the individual subunits of a RAR−RXR heterodimer proceeds independently.

Our results conflict with the report that suggested that within a DNA-bound RAR−RXR heterodimer, 9cRA is exclusively bound to RAR (Kurokawa *et al*., 1994). One possible explanation for this discrepancy is that the concentration of ligand used by Kurokawa *et al*. might have been such that only the RAR moiety within the dimers was saturated with ligand. Our data indicate that RAR binds 9cRA with a higher affinity than RXR, and that titration of a RAR−RXR heterodimer leads to sequential binding of 9cRA to the RAR subunit, followed by binding by the RXR subunit (Figure 4). Thus, if the saturation level of heterodimers in the studies of Kurokawa *et al*. was less than 50%, only ligands that bind to RAR, such as tRA or TTNPB, but not LG1069, would have effectively competed with 9cRA on binding. However, even if this interpretation is correct, an apparent discrepancy remains between our data and the finding that RAR diminishes binding of [^{3}H]LG1069 to RXR. It is also difficult to provide an explanation for the discrepancy between our data and the findings of Forman *et al*. (1995a). The data presented here were obtained using RXRα and RARα that lack the N-terminal A/B domain, while Forman *et al*. used GST-fusion constructs of the full-length receptors. It is unlikely, however, that the discrepancy stemmed from the usage of different protein constructs. This would suggest either thay the N-terminal A/B domain has an important, and as yet undescribed, function in ligand binding or that the GST-fusion construct possesses novel ligand-binding properties. Neither of these possibilities are very plausible. Finally, and maybe most important, we believe that optical methodologies of the type used here are a better approach to studying binding of hydrophobic ligands to proteins. Such methodologies can directly provide information on the stoichiometry of ligand binding, and they have the advantage that they do not require separation of free from bound ligand and, thus, unlike binding assays utilizing radioactive ligands, do not perturb the equilibrium attained by the system, and may report more accurately on equilibrium situations. For example, the data presented above suggest that the affinity of the ligand SR11246 for RXR is severalfold lower than that of 9cRA. This finding is in agreement with the observation that SR11246 is a less potent transactivator than 9cRA (Dawson *et al*., 1995). However, competition binding assays utilizing [^{3}H]-9cRA

showed similar binding affinities of 9cRA and SR11246 for RXRα (Dawson, unpublished data).

Given that formation of RAR−RXR heterodimers does not modify the ligand-binding properties of RXR, the findings that the RXR-selective ligand LG1069 did not transactivate RAR−RXR heterodimers remain unexplained. It was, however, reported that RAR and RXR within heterodimers respond to their ligands independently and in an additive fashion (Durnad *et al*., 1994), that LG1069 is an effective inhibitor of activation-induced apoptosis in T cells (Yang *et al*., 1995), and that a maximal effect requires the presence of ligands for both RAR and RXR (Bissonnette *et al*., 1995). These findings demonstrate that, at least in some settings, RAR−RXR heterodimers respond to ligands for both subunits. Further research is clearly needed to resolve these apparently conflicting observations.

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