The Isolation and Characterization of Purified Heterocomplexes of Recombinant Retinoic Acid Receptor and Retinoid X Receptor Ligand Binding Domains†

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ABSTRACT: Retinoic acid exerts many of its biological effects by interaction with heterocomplexes of nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). To further examine this interaction, a glutathione *S*-transferase (GST) fusion protein containing the ligand binding domain of human RXRα has been used to copurify the ligand binding domain of human RARγ by affinity chromatography over glutathione−agarose. Complexes of recombinant RAR−RXR ligand binding domains retaining full ligand binding capacity were purified, and their interactions with various retinoids were characterized by fluorometric titration and photoaffinity labeling. Analyses of the distribution of limiting amounts of [^{3}H]-*all-trans*-retinoic acid between cytoplasmic retinoic acid binding proteins, CRABP-I and CRABP-II, and the purified heterocomplexes indicate that *all-trans*-retinoic acid binds with comparable affinity to CRABP-I and the heterocomplexes, but with approximately 10-fold less affinity to CRABP-II. The aromatic retinoid acitretin, which is used in the treatment of psoriasis, binds relatively poorly to the purified heterocomplexes, although it binds with high affinity to the CRABPs. Acitretin displaces [^{3}H]-*all-trans*-retinoic acid from the CRABPs and increases retinoic acid occupancy of the heterocomplexes. These results suggest that certain retinoids could potentially perturb the distribution of endogenous retinoic acid between the CRABPs and the nuclear receptors and thus affect retinoid signaling. The purified recombinant complexes should provide a useful model system for further structural analysis of the dimerization interface between the RAR and RXR ligand binding domains.

Retinoic acid, as well as a number of its synthetic analogs, has profound effects on growth and differentiation and is used for the treatment of severe dermatological diseases and certain malignancies (Goodman, 1994; Hong & Itri, 1994; Peck & DiGiovanna, 1994). The retinoic acid receptors (RARs)[1] and retinoid X receptors (RXRs) are retinoic acid dependent transcriptional regulators which mediate many of the biological effects observed with administration of vitamin A (Mangelsdorf & Evans, 1995). The RARs (Petkovich *et al*., 1987; Giguère *et al*., 1987) bind both 9-*cis*-retinoic acid and *all-trans*-retinoic acid, while the RXRs (Hamada *et al*., 1989; Mangelsdorf *et al*., 1990, 1992; Leid *et al*., 1992) bind only 9-*cis*-retinoic acid (Heyman *et al*., 1992; Levin *et al*., 1992). The *all-trans*-retinoic acid occupancy of the RARs may be modulated by two cytoplasmic proteins, cellular retinoic acid binding protein-I (CRABP-I) and cellular retinoic acid binding protein-II (CRABP-II) (Li & Norris, 1996). These proteins also bind *all-trans*-retinoic acid with high affinity and have been proposed to limit nuclear levels of *all-trans*-retinoic acid by sequestration and/or promotion of *all-trans*-retinoic acid metabolism (Fiorella & Napoli, 1994; Boylan & Gudas, 1991, 1992).

The RXRs bind as homodimers or higher order oligomers to DNA response elements consisting of directly repeating hexamer purine-G(G/T)TCA sequences separated by one base pair. The RARs bind as heterodimers with RXR to DNA response elements that consist of direct repeats separated by one, two, or five base pairs. The RARs and RXRs are approximately 50 kDa in size and display a similar modular structure consisting of six regions (denoted A−F), including a DNA binding domain (denoted C) and a ligand binding domain (denoted E). Homodimeric and heterodimeric complex formation occurs via dimerization interfaces in the DNA binding domains and in the ligand binding domains (Zhang *et al*., 1992; Rosen *et al*., 1993; Zechel *et al*., 1994; Perlmann *et al*., 1996). Because RXRs can potentially form either homodimers or heterodimers when mixed with RARs, the physical isolation of purified RAR−RXR heterocomplexes from mixtures of the two receptors is important prior to further characterization of their properties. The isolation of RAR−RXR heterocomplexes would greatly aid in understanding the structural basis of the interactions between these two receptors and with their ligands.

In the current work, we have focused on the interactions between the ligand binding domains of RAR and RXR, by employing a GST−hRXRαDEF fusion protein (Cheng *et al*., 1994) to copurify recombinant hRARγDE domains by

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[1] Abbreviations: hRXRα, human retinoid X receptor α; hRARγ, human retinoic acid receptor γ; CRABP, cellular retinoic acid binding protein; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; K_d', apparent equilibrium dissociation constant; tRA, *all-trans*-retinoic acid; 9cRA, 9-*cis*-retinoic acid.

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affinity chromatography over glutathione−agarose. We report here the isolation of hRARγDE−hRXRαDEF complexes retaining full ligand binding capacity and the characterization of their interactions with natural and synthetic retinoids.

EXPERIMENTAL PROCEDURES

Materials. *all-trans*-Retinoic acid was obtained from Kodak. [11,12-^{3}H(N)]-*all-trans*-Retinoic acid (49.3 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE). [11,12-^{3}H(N)]-9-*cis*-Retinoic acid (54 Ci/mmol), unlabeled 9-*cis*-retinoic acid, and acitretin [*all-E*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid] were provided by Hoffmann-LaRoche. The RXR-selective ligand LG1069, 4-[(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethenyl]benzoic acid (Boehm *et al*., 1994), was a kind gift of Dr. Tim Willson (Glaxo). Thrombin, glutathione−agarose, glutathione (reduced form), and 13-*cis*-retinoic acid were from Sigma. All procedures involving retinoids were performed under dim or red light.

Expression and Purification of Recombinant Retinoic Acid Binding Proteins. Recombinant mouse CRABP-I and CRABP-II were expressed, purified, delipidated, and quantitated as described previously (Norris *et al*., 1994). The construction of GST−hRXRαDEF (amino acids 198−462) fusion protein expression vectors was described previously (Cheng *et al*., 1994). The truncated hRARγDE (amino acids 151−421) cDNA, incorporating a 5′ *Nde*I site and a 3′ *Bam*HI site, was amplified from the full-length hRARγ cDNA (Saiki *et al*., 1988) using the upstream forward primer 5′-GCATATGTCCAAGGAAGCTGTGCG-3′ and the downstream reverse primer 5′-GCGATCCTTATTACATTTCAGGGTTCTCCAG-3′ by the polymerase chain reaction. The truncated RAR cDNA was digested with *Nde*I and *Bam*HI, and ligated into *Nde*I/*Bam*HI-digested pET29a (Novagen, Madison, WI).

Prokaryotic expression of the recombinant nuclear retinoic acid receptors was carried out in the *Escherichia coli* strain BL21(DE3) (*lon*-, *ompT*-) (Studier *et al*., 1990). The expression of the GST fusion proteins was carried out as described previously (Cheng *et al*., 1994). For expression of the truncated hRARγDE ligand binding domain, a 20 mL overnight culture grown in LB broth containing kanamycin (50 μg/mL) was inoculated into 1 L of LB broth containing kanamycin (50 μg/mL) and incubated at 37 °C until an OD_{600} of 0.8 was reached. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.15 mM, and the cultures were incubated at 25 °C for an additional 3 h. Cells were harvested by centrifugation and stored at −80 °C. Cells expressing GST−hRXRαDEF or hRARγDE were resuspended in 40 mL of lysis buffer (50 mM Tris-HCl, pH 7.8, 40 mM KCl, 2 mM β-mercaptoethanol, 1 mM EDTA, 10 μM $ZnCl_2$, 5% glycerol, 0.01% monothioglycerol, and 0.02% azide) per liter of culture at 4 °C. The cells were lysed by sonication (30% output, 50% duty, 30 min) on ice using a Branson Sonifier Model 250/450 (Branson Ultrasonics Corp., Danbury, CT). The lysates were clarified by centrifugation at 10000*g* for 30 min.

One hundred milliliters of crude bacterial soluble extract containing GST−RXRαDEF prepared from 2 L of culture was applied to 35 mL of glutathione−agarose beads. The beads were washed extensively with 2 L of lysis buffer at 4 °C. Then 500 mL of crude bacterial soluble extract containing hRARγDE peptide prepared from 8 L of culture was applied to the beads and washed with 2 L of lysis buffer. The glutathione-agarose beads were washed with 2 volumes of digestion buffer (lysis buffer with the addition of 0.15 M NaCl and 2.5 μM $CaCl_2$), and incubated with 50 units of human thrombin in 30 mL of digestion buffer at 4 °C for 5 h, following which the thrombin was inactivated by the addition of 1 mM benzamidine. Under these conditions, the hRXRαDEF peptides were cleaved from GST while bound to glutathione−agarose, and the cleaved RAR−RXR ligand binding domain complexes were recovered in the supernatant. The cleaved peptides were further purified by gel filtration over a 2.6 cm × 60 cm Superdex 200 (Pharmacia) column equilibrated with TKME-150 buffer (25 mM Tris-HCl, pH 7.9, 150 mM KCl, 2 mM β-mercaptoethanol, 1 mM EDTA) at a flow rate of 1.0 mL/min at 4 °C. The column was calibrated using the following molecular mass standards: cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa.

Fluorescence Measurements. Fluorescence measurements were made with a Photon Technology International spectrofluorometer equipped with constant-temperature cell holders. Samples were excited at 280 nm and monitored at 330 nm using slits widths of 2 and 5 nm, respectively. Ligand dissolved in ethanol was titrated into samples such that the final total ethanol concentration did not exceed 0.5% (v/v). Titration measurements and inner filter effect corrections were performed as previously described (Norris *et al*., 1994) at 25 °C in TKME-150 buffer. Ligand stock concentrations were calculated based on the following molar absorption coefficients: *all-trans*-retinoic acid, 45 000 M^{-1} cm^{-1} at 350 nm (Cogan *et al*., 1976); 9-*cis*-retinoic acid, 36 500 M^{-1} cm^{-1} at 343 nm (Redfern *et al*., 1993); acitretin, 43 300 M^{-1} cm^{-1} at 353 nm (Norris *et al*., 1994); and LG1069, 16 400 M^{-1} cm^{-1} at 364 nm (Boehm *et al*., 1994). Concentrations of stock receptor protein solutions were determined based on quantitative amino acid analysis as described previously (Li *et al*., 1987).

Binding stoichiometries and apparent dissociation constants, K_d', were determined by nonlinear regression fitting of the fluorescence data to an equation derived from simple binding theory as described previously (Norris *et al*., 1994).

Photolabeling of Receptor Subsites with [^{3}H]Retinoic Acid. Purified hRXRαDEF or hRARγDE−hRXRαDEF was equilibrated with [^{3}H]-*all-trans*-retinoic acid or [^{3}H]-9-*cis*-retinoic acid for 10 min at room temperature. Ten microliter aliquots of the mixture containing 7.5 nmol of each peptide plus 2 pmol (0.1 μCi) of [^{3}H]-*all-trans*-retinoic acid or 2 pmol (0.1 μCi) of [^{3}H]-9-*cis*-retinoic acid were moved to the wells of a polycarbonate, 96-well micro-sample plate (Pharmacia). The plate was exposed to a 365 nm UV light source (Mineralight UVSL-25, Ultraviolet Products Inc.) at a distance of 1−2 cm above the plate for 10 min. Incident UV radiation was estimated at 10^{-3} W/cm^2, based on readings taken with a radiometer (Model IL1700; International Light Inc., Newburyport, MA). Following UV light exposure, the samples were combined with SDS−PAGE sample buffer containing β-mercaptoethanol (Laemmli, 1970) and analyzed by SDS−PAGE using 15% polyacrylamide gels. The gels were stained with Coomassie blue, soaked in EN^{3}HANCE (NEN, Boston, MA), and fluorographed

using Kodak XAR-film (Eastman Kodak, Rochester, NY).

Partitioning of [^{3}H]-all-trans-Retinoic Acid between CRABP and hRARγDE−hRXRαDEF. Four picomoles of [^{3}H]-*all-trans*-retinoic acid (0.2 μCi) dissolved in ethanol was added to 5 nmol of CRABP-I or -II and mixed with either hRXRαDEF (5 nmol) or hRARγDE−hRXRαDEF (5 nmol of each peptide) in a final volume of 2.5 mL of TKME-150. Each sample was subject to size-exclusion chromatography over Superdex 200 equilibrated with TKME-150 buffer at a flow rate of 1 mL/min at 4 °C. The radioactivity of collected fractions (4 mL) was determined by liquid scintillation counting. In some experiments, 10 nmol of acitretin was added immediately prior to the addition of [^{3}H]-*all-trans*-retinoic acid.

Relative binding affinities were calculated from the relative amounts of radioactivity which coeluted with each species using eq 1 which is derived from the relevant binding partition function (Wyman & Gill, 1990).

$$\frac{K_C}{K_D} = \frac{C_{cpm}[D]}{D_{cpm}[C]} \quad (1)$$

The terms C_{cpm} and D_{cpm} represent the amounts of radioactivity coeluting with CRABP or dimer, respectively. K_C and K_D are the association constants for the binding of *all-trans*-retinoic acid to CRABP and dimer, respectively. Because of the high molar excess of protein over ligand in these experiments, the unliganded amounts of CRABP, [C], and dimer, [D], are virtually equal to their total concentrations. The relative amounts of tetramer and dimer were determined from the relative areas of their elution A_{280} profiles. Under these conditions, roughly equal amounts of tetramer and dimer eluted from the column. Of note, this ratio was changed little by the addition of substoichiometric amounts of [^{3}H]-*all-trans*-retinoic acid or by the addition of stoichiometric amounts of acitretin.

RESULTS

Purification of hRARγDE−hRXRαDEF Heterocomplexes. The GST−hRXRαDEF fusion protein described previously (Cheng *et al*., 1994) was used to isolate complexes of RAR−RXR ligand binding domains for further analysis. The GST−hRXRαDEF fusion protein from crude bacterial extracts was initially adsorbed to glutathione−agarose beads followed by adsorption of hRARγDE from crude bacterial extracts as described under Experimental Procedures. Complexes of hRARγDE−hRXRαDEF were eluted by thrombin cleavage of the matrix-bound GST−hRXRαDEF fusion protein. Approximately 20 mg of cleaved protein complex was recovered from 2 and 8 L of bacterial cultures expressing GST−hRXRαDEF and hRARγDE, respectively, in the column eluate. The protein complex was further fractionated by size-exclusion chromatography over Superdex 200 into four fractions (1−4) with relative A_{280} ratios of 2:1:6:6 (see Figure 1). Equivalent amounts of the hRARγDE and hRXRαDEF peptides were detected in fractions 2−4, as quantitated by scanning densitometry of Coomassie-stained SDS−polyacrylamide gels (see Figure 1). The only peptide detected in fraction 1 was hRXRαDEF. The estimated molecular mass of the protein in fraction 1 based on its retention time was 35 kDa, which corresponds to the predicted molecular mass of monomeric hRXRαDEF of 32 kDa. The estimated molecular mass of fraction 2 was 65 kDa, suggesting that the protein in this fraction consisted of dimeric complexes of receptor ligand binding domains. The estimated molecular mass of fraction 3 was 150 kDa, suggesting that the protein in this fraction consisted of tetrameric complexes of receptor ligand binding domains. Fraction 4 eluted in the void volume of the column, indicating that these complexes represented higher order multimers with a molecular mass greater than 600 kDa. Rechromatography of fraction 3 yielded complexes corresponding to both dimers and tetramers, suggesting that there was exchange between these two forms. Further dissociation to monomer subunits was not detected. The higher order multimers appeared to be irreversibly aggregated, since no dissociation to lower order complexes was observed on rechromatography of fraction 4. In contrast, size-exclusion chromatography of the hRXRαDEF peptide alone revealed that the peptide was predominantly in the monomer form (data not shown), consistent with previous equilibrium sedimentation experiments (Cheng *et al*., 1994).

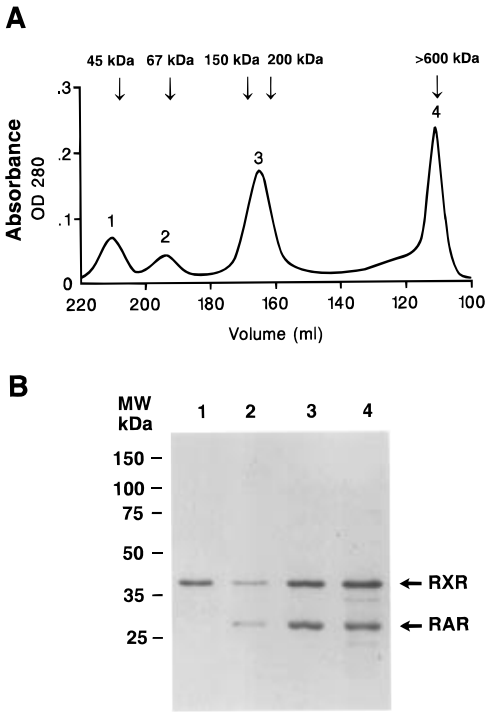

FIGURE 1: Size-exclusion chromatography of hRARγDE−hRXRαDEF complexes isolated by affinity chromatography. (A) The glutathione−Sepharose affinity column eluate, recovered after thrombin cleavage of adsorbed GST−hRXRαDEF fusion protein and hRARγDE, was loaded onto a Superdex 200 gel filtration column. Elution of proteins was monitored by in-line detection of sample A_{280}. The column was previously standardized with proteins of known molecular mass as shown. (B) Coomasie-stained SDS−10% polyacrylamide gel of proteins recovered in fractions 1−4.

Fluorometric Titration of hRARγDE−hRXRαDEF Complexes with Retinoids. Fluorescence quenching methods were utilized to assess the ligand binding activities of the hRARγDE−hRXRαDEF heterocomplexes recovered in fractions 2−4. The heterocomplexes exhibited intrinsic protein fluorescence with a maximum at 328 nm when excited at 290 nm, due to three tryptophans. Two tryptophans are contained in hRXRαDEF at positions 282 and 305, while one tryptophan is contained in hRARγDE at position 227. Very little change of protein fluorescence was observed when

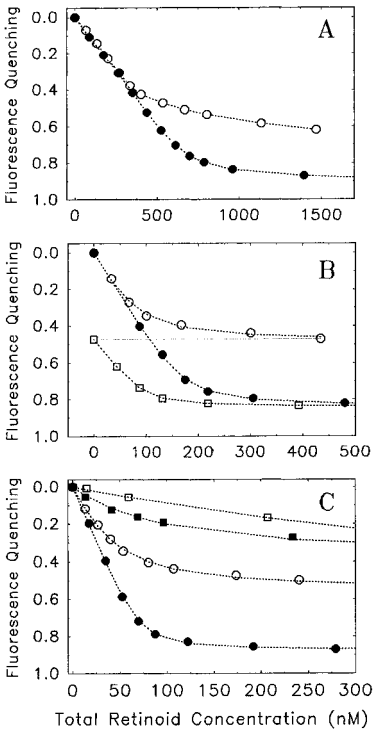

FIGURE 2: Fluorometric titration of hRARγDE−hRXRαDEF with 9-*cis*-retinoic acid and *all-trans*-retinoic acid. Samples were diluted in TKME-150 and titrated as described under Experimental Procedures. (Panel A) Complexes from fraction 3 (350 nM per subsite) titrated with 9-*cis*-retinoic acid (●) or *all-trans*-retinoic acid (○); (panel B) complexes from fraction 2 (100 nM per subsite) titrated with 9-*cis*-retinoic acid (●), *all-trans*-retinoic acid (○), or with 9-*cis*-retinoic acid (□) after titration with *all-trans*-retinoic acid; (panel C) complexes from fraction 3 (50 nM per subsite) were titrated with acitretin (■) or 13-*cis*-retinoic acid (□). For comparison, the titrations with *all-trans*-retinoic acid (○) and 9-*cis*-retinoic acid (●) are also shown. Dashed lines represent nonlinear fits.

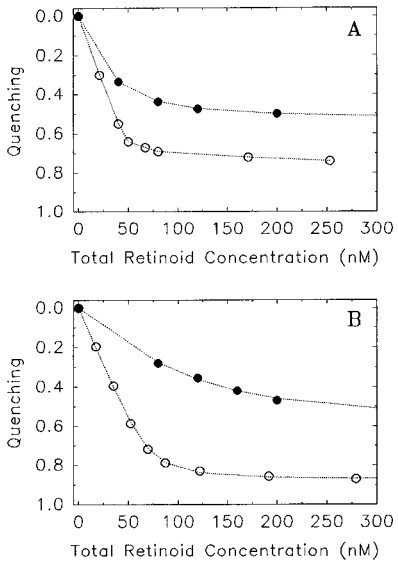

FIGURE 3: Fluorometric titration of hRXRαDEF and hRARγDE−hRXRαDEF with LG1069. (A) hRXRαDEF was diluted to 40 nM and titrated with LG1069 (●) as described under Experimental Procedures. (B) Heterocomplexes from fraction 3 were diluted to 50 nM per subsite and titrated with LG1069 (●). In both panels, titration with 9-*cis*-retinoic acid (○) is included for comparison.

increasing amounts of either *all-trans*-retinoic acid or 9-*cis*-retinoic acid were added to the complexes recovered in the void volume (i.e., fraction 4), indicating that these complexes were essentially inactive with respect to ligand binding (data not shown).

Fluorescence titrations of the hRARγDE−hRXRαDEF complexes recovered in fractions 2 or 3 with various retinoids yielded essentially the same binding curves, as shown in Figure 2. The addition of 9-*cis*-retinoic acid to the heterocomplexes recovered in fractions 2 and 3 resulted in a linear decrease in protein fluorescence until the ligand binding sites were saturated. At saturation, the binding of 9-*cis*-retinoic acid to the RAR−RXR complex decreased the total protein fluorescence by 80%. The number of binding sites for 9-*cis*-retinoic acid in the hRARγDE−hRXRαDEF complex was 0.94 ± 0.03 (n = 2) per receptor subsite, with a K_d' of 3 ± 1 nM ($n = 5$).

The addition of *all-trans*-retinoic acid to the hRARγDE−hRXRαDEF complexes recovered in fractions 2 or 3 also resulted in a linear decrease in protein fluorescence until saturation of the binding sites (Figure 2). However, at saturation, the binding of *all-trans*-retinoic acid decreased the total protein fluorescence by only ~40%. The number of binding sites for *all-trans*-retinoic acid in the hRARγDE−hRXRαDEF complex was 0.51 ± 0.07 ($n = 2$) per receptor subsite with a K_d' of 10 ± 7 nM ($n = 6$). This is consistent with *all-trans*-retinoic acid binding with high affinity only to the RAR subsite. Addition of 9-*cis*-retinoic acid to the hRARγDE−hRXRαDEF complex after saturation of the *all-trans*-retinoic acid binding sites led to additional decreases in protein fluorescence (Figure 2B). Based on an analysis of the titration curves, the number of binding sites for 9-*cis*-retinoic acid following titration with *all-trans*-retinoic acid was calculated to be 0.49 ± 0.01 ($n = 3$) with a K_d' of 5 ± 2 nM ($n = 3$). Thus, the heterocomplexes recovered in fractions 2 and 3 exhibited full ligand binding capacity.

Two synthetic retinoids used in the clinical practice of dermatology are isotretinoin (13-*cis*-retinoic acid) and acitretin, the acid derivative of etretinate (Peck & DiGiovanna). The addition of these retinoids to the purified recombinant heterocomplexes from fraction 3 is shown in Figure 2C and demonstrates little change of protein fluorescence. Similar results were obtained for complexes in fraction 2 (data not shown). This indicates that these two compounds bind poorly to the RAR−RXR heterocomplexes. While the mechanism of action for 13-*cis*-retinoic acid may involve isomerization to *all-trans*-retinoic acid and/or 9-*cis*-retinoic acid, the mechanism of action for acitretin is unclear since it appears unable to act directly upon the RAR−RXR complexes.

To examine whether association with the unligated RAR ligand binding domain prevented the RXR ligand binding domain from interacting with ligand, binding of an RXR selective ligand, LG1069 (Boehm *et al.*, 1994), to hRXRαDEF monomer was compared with binding to purified heterocomplex (Figure 3). Addition of LG1069 to the hRXRαDEF monomer led to saturable decreases in protein fluorescence (Figure 3A), with 1.1 ± 0.3 binding sites per monomer and a K_d' of 27 ± 17 nM ($n = 3$). Addition of LG1069 to the hRARγDE−hRXRαDEF heterocomplex also resulted in a decrease in protein fluorescence (Figure 3B), indicating that the RXR subunit is still capable of binding this ligand with a stoichiometry of 1.2 ± 0.5 when complexed with the unligated RAR subunit, but with a somewhat reduced affinity, K_d' of 55 ± 16 nM ($n = 3$) derived from analysis of the titration curves.

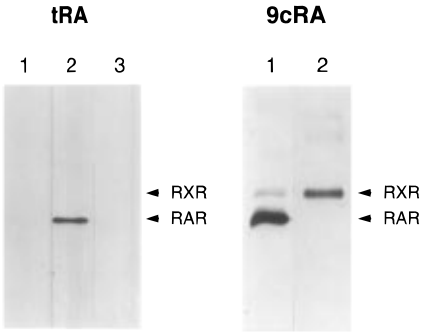

FIGURE 4: Photoaffinity labeling of hRARγDE−hRXRαDEF with [^{3}H]retinoic acid. Fluorographs of samples analyzed by SDS−10%polyacrylamide gel electrophoresis are shown. The positions of hRXRαDEF (RXR) and hRARγDE (RAR) peptides are indicated by the arrows. (Left panel, tRA) hRARγDE−hRXRαDEF complexes (fraction 3) were labeled with [^{3}H]-*all-trans*-retinoic acid by the photoaffinity technique described under Experimental Procedures. (Lane 1) hRARγDE−hRXRαDEF complex and [^{3}H]-*all-trans*-retinoic acid unexposed to light; (lane 2) hRARγDE−hRXRαDEF complex and [^{3}H]-*all-trans*-retinoic acid exposed to UV light for 10 min; (lane 3) hRARγDE−hRXRαDEF complex, [^{3}H]-*all-trans*-retinoic acid, and a 375-fold molar excess of unlabeled *all-trans*-retinoic acid exposed to UV light for 10 min. (Right panel, 9cRA) Photoaffinity labeling of hRARγDE−hRXRαDEF complex and (lane 2) hRXRαDEF monomer with [^{3}H]-9-*cis*-retinoic acid. Equivalent amounts of the RXR subunit (7.5 nmol) were included in each sample.

Photoaffinity Labeling of Binding Subsites in hRARγDE−hRXRαDEF Heterocomplexes. The localization of bound *all-trans*-retinoic acid and 9-*cis*-retinoic acid within the heterocomplexes could be further characterized by photoaffinity labeling. *all-trans*-Retinoic acid has been reported to specifically photolabel CRABP and albumin in complex mixtures of cytosolic proteins (Bernstein *et al*., 1995). As shown in Figure 4, photolabeling of the RAR subsite in the hRARγDE−hRXRαDEF heterocomplex with [^{3}H]-*all-trans*-retinoic acid was observed. The labeling occurred with an estimated efficiency of 20% and required UV light exposure. It was greatly reduced by the addition of a 375-fold molar excess of unlabeled *all-trans*-retinoic acid (see Figure 4).

To determine whether 9-*cis*-retinoic acid binds initially to the RAR or RXR subsite, a substoichiometric amount of [^{3}H]-9-*cis*-retinoic acid was added to the hRARγDE−hRXRαDEF heterocomplex and exposed to UV light. As shown in Figure 4, there was preferential photolabeling of the RAR subsite within the heterocomplex, and the amount incorporated in the RXR subsite within the heterocomplex was reduced approximately 70% compared to the amount incorporated into an equivalent amount of RXR monomer. With the addition of increasing amounts of [^{3}H]-9-*cis*-retinoic acid, the difference in the amount of label incorporated into monomeric hRXRαDEF compared to the heterocomplexes was eliminated (data not shown).

Partitioning of [^{3}H]-all-trans-Retinoic Acid between CRABPs and the RAR−RXR Complexes. Size exclusion chromatography of purified proteins mixed with substoichiometric amounts of [^{3}H]-*all-trans*-retinoic acid was used to directly compare the relative binding affinities of *all-trans*-retinoic acid for the CRABPs and the purified nuclear receptor complexes. As shown in Figure 5, when [^{3}H]-*all-trans*-retinoic acid was added to a mixture comprised of equal binding equivalents of CRABP-II and the hRARγDE−hRXRαDEF complex, 8 ± 3% (n = 2) of the total radioactivity was recovered in the CRABP-II fraction, 76 ± 3% was recovered in the hRARγDE−hRXRαDEF "dimeric" fraction, and 2 ± 2% was recovered in the hRARγDE−hRXRαDEF "tetrameric" fraction. When [^{3}H]-*all-trans*-retinoic acid was added to equal binding equivalents of CRABP-I and the hRARγDE−hRXRαDEF complex, 49 ± 8% (n = 2) of the total radioactivity was recovered in the CRABP-I fraction, 38 ± 8% was recovered in the hRARγDE−hRXRαDEF "dimeric" fraction, and 1.5 ± 0.4% in the "tetrameric" fraction. When either CRABP was mixed with hRXRαDEF monomer, nearly all of the loaded radioactivity was recovered in the CRABP-I fraction (83%) or in the CRABP-II fraction (86%) with less than 2% of the radioactivity associated with the hRXRαDEF monomer.

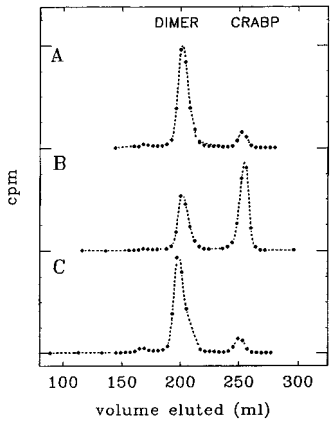

FIGURE 5: Partitioning of [^{3}H]-*all-trans*-retinoic acid between the CRABPs and hRARγDE−hRXRαDEF complexes. Size-exclusion chromatography was used to analyze partitioning of [^{3}H]-*all-trans*-retinoic acid (4 pmol) between (A) CRABP-II (5 nmol) and hRARγDE−hRXRαDEF (5 nmol each subunit); (B) CRABP-I (5 nmol) and hRARγDE−hRXRαDEF (5 nmol each subunit); (C) CRABP-I (5 nmol), hRARγDE−hRXRαDEF (5 nmol each subunit), and acitretin (10 nmol). The positions at which the hRARγDE−hRXRαDEF dimer and CRABP elute are as shown.

The high recovery of total radioactivity loaded onto the Superdex column indicates that there is little dissociation of the protein−ligand complexes during fractionation by size-exclusion chromatography. The absence of radioactivity associated with the hRXRαDEF monomer indicates that the level of nonspecific association of [^{3}H]-*all-trans*-retinoic acid with proteins is very low in this assay. The relative affinity of the dimeric hRARγDE−hRXRαDEF complex for *all-trans*-retinoic acid is calculated to be (17 ± 5)-fold (n = 2) higher than the affinity of CRABP-II and (1.3 ± 0.1)-fold higher than the affinity of CRABP-I for *all-trans*-retinoic acid at 4 °C, using eq 1. There was preferential association of *all-trans*-retinoic acid with the dimeric form over the tetrameric form of the heterocomplexes, indicating that the dimeric complex has a much higher affinity for *all-trans*-retinoic acid than the tetrameric complex. Consistent with this, the addition of a stoichiometric amount of either *all-trans*-retinoic acid or 9-*cis*-retinoic acid to the RAR−RXR complex results in complete disappearance of the tetrameric species (data not shown).

Acitretin Increases the Occupancy of the RAR−RXR Complex by Displacement of [^{3}H]-all-trans-Retinoic Acid from CRABP-I. Acitretin, a synthetic aromatic retinoid analog, is used in the treatment of severe psoriasis. Its use is limited by extreme teratogenicity. The above fluorescence studies indicate that acitretin binds relatively poorly to the hRARγDE−hRXRαDEF complex, while previous fluores-

cence studies have shown that it binds to CRABP-I and CRABP-II with high affinity (Norris *et al*., 1994). A mechanism by which acitretin exerts its biological effects may involve an indirect signaling pathway by displacing endogenous retinoic acid complexed with the CRABPs (Norris *et al*., 1994). The effect of acitretin on the partitioning of [^{3}H]-*all-trans*-retinoic acid between CRABP-I and hRARγDE−hRXRαDEF was therefore examined. As shown in Figure 5, there was a dramatic shift in the distribution of radioactivity between CRABP-I and hRARγDE−hRXRαDEF complex upon the addition of a stoichiometric amount of acitretin such that 82 ± 5% (n = 2) of the radioactivity eluted with the dimeric form of the complex. The amount of radioactivity associated with CRABP-I was reduced to 14 ± 5% (n = 2). Similarly, acitretin reduced the amount of radioactivity associated with CRABP-II (from 69% to 28%) and increased the amount associated with the hRARγDE−hRXRαDEF complex (from 17% to 57%) in a mixture containing 25 nmol of CRABP-II and 2.5 nmol of hRARγDE−hRXRαDEF. The addition of acitretin thus increased *all-trans*-retinoic acid occupancy of the nuclear receptor by displacing *all-trans*-retinoic acid from the cytoplasmic binding proteins.

DISCUSSION

We have purified heterocomplexes of hRARγDE with hRXRαDEF which retain full ligand binding capacity. RXR can potentially form homodimers as well as heterodimers with RARs when the two receptors are mixed together. We have therefore taken steps to further fractionate the complexes by affinity chromatography and size-exclusion chromatography. We found that the purified heterocomplexes exhibited heterogeneity with respect to oligomerization states and binding capacity. Approximately 50% of the heterocomplexes were recovered after affinity chromatography as dimers and tetramers which retained full ligand binding capacity as measured by fluorescence quenching methods. Dissociation of the tetrameric species to the dimeric species was observed on rechromatography of fraction 3, suggesting that the two forms are in equilibrium. The remaining half were recovered as very large oligomers (>600 kDa) which are inactive with respect to ligand binding.

The active forms of the purified recombinant heterocomplexes exhibited half of the number of binding sites for *all-trans*-retinoic acid as for 9-*cis*-retinoic acid as predicted. Analysis of ligand binding by the heterocomplexes was complicated by the linkage between ligation and the oligomerization state of the heterocomplexes, as evidenced by the disappearance of the tetrameric forms on the addition of a stoichiometric amount of *all-trans*-retinoic acid or 9-*cis*-retinoic acid as determined by size-exclusion chromatography. A similar dissociation of tetramers comprised of *E. coli*-derived RXRα lacking the N-terminal A/B domain (RXR$\alpha\Delta$AB) was observed with ligation (Kersten *et al*., 1995). In contrast, *E. coli*-derived hRARα, which existed primarily as monomers in the absence of ligand as determined by size-exclusion chromatography, formed homodimers after binding *all-trans*-retinoic acid (Lefebvre *et al*., 1995). While the linkage of ligation with changes in oligomerization states suggests that the RAR−RXR ligand binding domain complexes are allosteric systems, it is difficult to predict the physiological significance of the various oligomerization states observed for the recombinant heterocomplex of RAR and RXR ligand binding domains. The aggregation of recombinant truncated receptors is affected by relatively minor changes such as the degree of receptor truncation, growth conditions for the bacterial cultures, and the specific fusion peptide used (Cheng *et al*., 1994; Berggren Söderlund *et al*., 1995; Lupsiella *et al*., 1995). For example, 50% of purified histidine-tagged hRXRαDEF (amino acids 200−462) peptide (Bourguet *et al*., 1995a,b) is recovered as homodimers, whereas hRXRαDEF (amino acids 198−462) peptide obtained by thrombin cleavage of the GST-fusion protein is recovered primarily as monomers. We have observed that heterocomplexes formed between the hRXRαDEF monomer and recombinant hRARγDE protein fused with the 21 amino acid peptide containing a histidine-tag precipitated at relatively low protein concentrations.[2]

The localization of bound *all-trans*-retinoic acid and 9-*cis*-retinoic acid within the complex was further characterized by photoaffinity labeling. Photoaffinity labeling of the purified heterocomplexes with [^{3}H]-*all-trans*-retinoic acid exclusively labeled the RAR subsite as predicted. With the addition of substoichiometric amounts of [^{3}H]-9-*cis*-retinoic acid to the heterocomplexes, there was initially preferential labeling of the RAR subsite over the RXR subsite. This is consistent with previous reports that the RARs exhibit a higher binding affinity for 9-*cis*-retinoic acid than do the RXRs (Heyman *et al*., 1992; Levin *et al*., 1992; Allenby *et al*., 1993; Allegretto *et al*., 1993). Alternatively, there may be allosteric interactions between the unliganded RAR that prevent binding of ligand to the RXR subsite (Kurokawa *et al*., 1994; Forman *et al*., 1995), although this has not been observed by other investigators (Kersten *et al*., 1996). Fluorescence studies indicate that the RXR-selective ligand LG1069 binds to the RXR subsite when associated with unliganded RAR but with somewhat reduced affinity. These results indicate that while association with the unligated RAR ligand binding domain does not prevent binding to the RXR subsite, there does appear to be a quantitative effect on the affinity of the RXR subsite due to interaction between the two subsites, independent of receptor interactions with DNA.

The mechanism by which retinoic acid is photochemically linked to proteins remains to be determined, but it is presumed that free radical species as well as photooxidation products such as the 5,6-epoxide are formed when retinoic acid is photodecomposed (Bernstein *et al*., 1995). X-ray analysis of crystalline recombinant hRARγDE reveals the presence of a number of amino acid residues containing functional groups that could potentially react with intermediates produced by photodecomposition of bound *all-trans*-retinoic acid, such as Cys-227 (Renaud *et al*., 1995). As RARγ is the predominant retinoic acid receptor subtype in human epidermis (Fisher *et al*., 1994), the observation that hRARγDE can be photolabeled by light of modest intensity may potentially be of physiological significance.

The dissociation constants of the cytoplasmic and nuclear retinoid binding proteins as determined by fluorometric titration represent only an upper estimate (Norris *et al*., 1994; Kersten *et al*., 1996). We have directly compared the relative binding affinities of the recombinant nuclear and cytoplasmic retinoic acid binding proteins by measuring the distribution

[2] K. Tian and E. Li, unpublished observations.

of a substoichiometric amount of [^{3}H]-*all-trans*-retinoic acid between the proteins. These experiments demonstrate that at 4 °C, *all-trans*-retinoic acid binds with comparable affinity to the nuclear receptor heterocomplexes and CRABP-I. This is consistent with the observation that increases in CRABP-I levels are correlated with decreases in retinoic acid-dependent transactivation (Boylan & Gudas, 1991).

Our studies demonstrate that *all-trans*-retinoic acid binds to the nuclear receptor heterocomplexes with ca. 10-fold higher affinity than to CRABP-II. By extrapolation of these data, the binding affinity of CRABP-I for *all-trans*-retinoic acid is estimated to be ca. 10-fold higher than that of CRABP-II, which is consistent with a number of previous studies which report that CRABP-I binds *all-trans*-retinoic acid with higher affinity than does CRABP-II (Li & Norris, 1996). These results suggest that CRABP-I will more effectively sequester *all-trans*-retinoic acid from the nuclear receptors than will an equivalent amount of CRABP-II.

Fluorescence titration studies indicate that acitretin binds poorly to purified hRARγDE−hRXRαDEF complexes but binds with high affinity to the CRABPs (Norris *et al*., 1994). This is consistent with previous studies demonstrating poor binding of acitretin to the recombinant RARβ ligand binding domain (Berggren Söderland *et al*., 1995), as well as poor transactivation of RARs in CV-1 cells by acitretin (Åström *et al*., 1990). The mechanism by which acitretin exerts its biological effect may therefore differ significantly from the retinoids that directly transactivate the RARs.

We have shown that acitretin induces a significant shift of *all-trans*-retinoic acid from CRABP-I and CRABP-II to hRARγDE−hRXRαDEF dimers, suggesting that acitretin could perturb the distribution of endogenous retinoic acid between the cytoplasmic and nuclear compartments of the cell and thus indirectly affect retinoid signaling. Whether the displacement of CRABP-bound retinoic acid by acitretin observed in vitro has physiological relevance in vivo remains to be determined. Of note, both CRABPs are expressed at high levels in the epidermis (Åström, 1994; Siegenthaler *et al*., 1992), and the therapeutic acitretin levels achieved in the skin are considerably higher than *all-trans*-retinoic acid levels (Siegenthaler *et al*., 1992). The responsiveness of tissue culture lines to acitretin may correlate with the presence or absence of CRABP. HL-60 promyelocytic leukemia cells, which differentiate in response to retinoic acid but not in response to acitretin (Chomienne *et al*., 1986), contain no detectable CRABP (Nervi *et* al., 1989), and F9 embryonal carcinoma cells, which do differentiate in response to acitretin, contain CRABP (Bailly *et al*., 1990). Although overexpression of CRABP-I reduces retinoic acid responsiveness in F9 cells (Boylan & Gudas, 1991), somewhat contrary results were observed in primary cultures of murine embryonic palate cells (Nugent & Greene, 1995), and no consistent effect was observed in COS-1 cells that were also overexpressing RAR and RAR−RXR (Venepally *et al*., 1996). Mutant mice lacking CRABP-I and/or CRABP-II (de Bruijn *et al*., 1994; Gorry *et al*., 1994; Fawcett *et al*., 1995; Lampron *et al*., 1995) probably represent the most definitive experimental system for examining whether the CRABPs play a significant role in acitretin signaling and toxicity. While these mice have been shown not to have increased susceptibility to the teratogenic effects of excess retinoic acid, they may exhibit reduced susceptibility to the teratogenic effects of acitretin.

The copurification of recombinant hRARγDE with the GST−hRXRαDEF fusion protein indicates that these two receptors interact strongly via their ligand binding domains. This observation coupled with the observation that the hRXRαDEF peptide exists predominantly in monomeric form (Cheng *et al*., 1994) suggests that association of RXR with RAR is favored over RXR self-association at the ligand binding domain interface. This is consistent with previous coimmunoprecipitation experiments reported by Marks *et al*. (1992) and cotransfection experiments with GAL4 and VP-16 hybrid fusions with the ligand binding domains of RAR and RXR reported by Perlmann *et al*. (1996). The strong interactions between the RAR and RXR ligand binding domains probably represent the structural basis for the dominant repression of retinoic acid-dependent transactivation by truncated RXRβ (Minnuci *et al*., 1994; Bases *et al*., 1994) and RARβ (Shen *et al*., 1993) receptors lacking the DNA binding domains.

The dimerization of the interface between RXR ligand binding domains has been described (Bourget *et al*., 1995a). The structural basis for the interactions between the RXR and RAR ligand binding domains remains to be precisely defined. Analysis of truncated and chimeric receptors suggests that the homo- and heterodimerization surfaces of RXR are not completely identical (Zhang *et al*., 1994; Leng *et al*., 1995; Perlmann *et al*., 1996). Structural analysis of these purified recombinant heterocomplexes may provide further insight regarding the heterodimerization interface between the RXR and RAR ligand binding domains.

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