Characterization of the Interaction between Retinoic Acid Receptor/Retinoid X Receptor (RAR/RXR) Heterodimers and Transcriptional Coactivators through Structural and Fluorescence Anisotropy Studies*

Received for publication, August 13, 2004, and in revised form, October 21, 2004. Published, JBC Papers in Press, November 4, 2004, DOI 10.1074/jbc.M409302200

Vivian Pogenberg†, Jean-François Guichou‡, Valérie Vivat-Hannah†, Sabrina Kammerer**, Efrén Pérez‡‡, Pierre Germain†§, Angel R. de Lera‡‡, Hinrich Gronemeyer†, Catherine A. Royer‡, and William Bourguet$$$ From the †Centre de Biochimie Structurale, CNRS U5048-INSERM U554-UM1, Faculté de Pharmacie, 15 avenue Charles Flahaut, 43093 Montpellier, France, the Departments of §Cell Biology and Signal Transduction and $$Structural Biology and Genomics, Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 rue Laurent Fries, B. P. 10142, 67404 Illkirch, France, and the ‡‡Departamento de Quimica Organica, Facultad de Quimica, Universidade de Vigo, 36200 Vigo, Spain.

Retinoid receptors (RARs and RXRs) are ligand-activated transcription factors that regulate the transcription of target genes by recruiting coregulator complexes at cognate promoters. To understand the effects of heterodimerization and ligand binding on coactivator recruitment, we solved the crystal structure of the complex between the RAR/RRXα ligand-binding domain heterodimer, its 9-cis retinoic acid ligand, and an LXXLL-containing peptide (termed NR box 2) derived from the nuclear receptor interaction domain (NID) of the TRAP220 coactivator. In parallel, we measured the binding affinities of the isolated NR box 2 peptide or the full-length NID of the coactivator SRC-1 for retinoid receptors in the presence of various types of ligands. Our correlational analysis of three-dimensional structures and fluorescence data reveals that heterodimerization does not significantly alter the structure of individual subunits or their intrinsic capacity to interact with NR box 2. Similarly, we show that the ability of a protomer to recruit NR box 2 does not vary as a function of the ligand binding status of the partner receptor. In contrast, the strength of the overall association between the heterodimer and the full-length SRC-1 NID is dictated by the combinatorial action of RAR and RXR ligands, the simultaneous presence of the two receptor agonists being required for highest binding affinity. We identified an LXXLL peptide-driven mechanism by which the concerted reorientation of three phenylalanine side chains generates an “aromatic clamp” that locks the RXR activation helix H12 in the transcriptionally active conformation. Finally, we show how variations of helix H11-ligand interactions can alter the communication pathway linking helices H11, H12, and the connecting loop L11–12 to the coactivator-binding site. Together, our results reveal molecular and structural features that impact on the ligand-dependent interaction of the RAR/RXR heterodimer with nuclear receptor coactivators.

Retinoids are the active metabolites of vitamin A that regulate complex gene networks involved in cell differentiation, proliferation, and apoptosis (1–4). Notably, they are effective inhibitors of tumor cell growth, thus supporting their actual or potential use in cancer therapy and prevention (5–7). The biological activity of all-trans and 9-cis retinoic acid (9C-RA)1 signals are mediated by two families of nuclear receptors that act as ligand-dependent transcriptional regulators: the retinoic acid receptors (RARα, -β, and -γ) and the retinoid X receptors (RXRα, -β, and -γ). Although RARs bind and are activated by both the 9-cis and the all-trans isomers of the retinoic acid, RXRs are exclusively activated by the 9-cis isomer (8–10). Like other members of the nuclear receptor superfamily, retinoid receptors are modular and include an N-terminal A/B activation domain, a central DNA-binding domain (region C), a linker region D, a ligand-binding domain (region E), and a C-terminal region F of unknown structure and function. The ligand-binding domain (LBD) is a multifunctional domain, capable of ligand binding, dimerization, and interaction with transcriptional coregulators.

Previous studies have shown that retinoid actions are predominantly mediated through the formation of heterodimers between the various RAR and RXR isotypes. The RAR/RXR heterodimers can operate as either transcriptional repressors or activators. The silencing activity involves the establishment of transcription repressing complexes in which corepressors are recruited to receptor heterodimers in the absence of ligand (apo-receptors) (11–15). Binding of agonists (holo-receptors)...

---

* This work was supported by funds from the INSERM, the CNRS, the Université Montpellier I, the European Commission (Grant RTD QLK3-2002-02029 “Anticancer Retinoids”), the Spanish MCYT (Grant SAF01-3288), and the Xunta de Galicia (Grant PGIDIT02PXIC30108PN). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “costs of publication of this article were defrayed in part by the payment of page charges.” This article must therefore be hereby marked “costs of publication of this article were defrayed in part by the payment of page charges.”

† The recipient of a grant from the Réseau National des Génopoles, Ministère de la Recherche et de la Technologie.

‡ Current address: Aix S, A Bioinstitut de l’ESBS, Boulevard Sebastien Brandt, 67404 Illkirch, France.

§§ To whom correspondence should be addressed. Tel.: 33-0-4-67-04-34-34; Fax: 33-0-4-67-52-96-23; E-mail: bourguet@cbn.cnrs.fr.

1 The abbreviations used are: 9C-RA, 9-cis retinoic acid; RXR, retinoid X receptor; RAR, retinoic acid receptor; NR box, nuclear receptor box; NID, nuclear receptor interaction domain; LBD, ligand-binding domain; AF-2, activation function 2; TRAP220, thyroid receptor-associated protein 220; SRC-1, steroid receptor coactivator 1; r.m.s.d., root mean square deviation; TTNPB, ethyl-p-[(E)-2(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-l-propenyl]-benzoic acid; CAT, chloramphenicol acetyltransferase.
triggers a mechanism by which the most C-terminal LBD helix H12, encompassing the core of the functionally conserved activation function 2 (AF-2), is repositioned in a so-called active- or holo-conformation, thereby creating a binding surface that facilitates interaction with coactivator proteins and concomitantly decreases the affinity for corepressors (16–18). Conversely, binding of antagonists prevents the formation of this specific surface by interfering directly or indirectly with the holo-conformation of helix H12 (16, 19). Transcriptional coactivators including those of the SRC-1/p160 family and the TRAP complex are involved in chromatin remodeling or recruitment of the basal transcription machinery and interact with nuclear receptors via short LXXLL helical motifs, so-called NR boxes, present as multiple copies in their nuclear receptor interaction domain (NID) (20, 21). The ligand-induced exchange of corepressor and coactivator complexes is believed to underlie the molecular mechanism controlling target gene repression and activation (22, 23).

In an effort to gain new insights into the molecular mechanism of the RAR/RXR-coactivator interaction, we solved the crystal structure of the RARβ/RXRα LBD heterodimer in complex with 9C-RA and a 13-residue fragment containing the NR box 2 of the coactivator TRAP220. Furthermore, we used fluorescence anisotropy to characterize in a quantitative manner the interaction between the transcriptional coactivator SRC-1 and monomeric or heterodimeric retinoid receptors bound to a variety of ligands. Based on these structural and biophysical studies, we propose a model of the RAR/RXR-SRC-1 interaction to understand how dimerization and ligand binding influence coactivator recruitment.

**EXPERIMENTAL PROCEDURES**

**Peptides and Ligands**—The human SRC-1<sub>1-780</sub> coactivator fragment was purified and labeled with Alexa Fluor 488 as described (24). Fluorescent peptide (fluorescein-β-A<sub>6</sub>D/HKHLRQEGS699) corresponding to the NR box 2 binding motif of SRC-1 was purchased from NeoSystem (Strasbourg, France). Unlabeled peptides corresponding to NR box 1 (H9251-TSHVL/LQLTITTA424), NR box 2 (H9252-HKL/RQEGS695), NR box 3 (D/HQDLYL/LKDB296) of SRC-1, and NR box 4 of human TRAP220 coactivator (V341/PMPLRKGLIAKPA629) were prepared on a Pioneer peptide synthesis system from Applied Biosystems (Solid Phase Peptide Synthesis) using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) activation. BMS614 and CD3254 were kindly provided by Montpellier, France). BMS614 and CD3254 were kindly provided by

**PAGE**, pooled, and mixed with 2-fold molar excess of 9C-RA and 3-fold molar excess of TRAP220 NR box 2 peptide (641NHPMLMNLLKD-NPA<sup>654</sup>). The purified complex was concentrated to 15 mg/ml-1 and centrifuged for 30 min at 13,000 rpm prior to crystallization assays. Crystals with unit cell parameters a = b = 115.68 Å and c = 247.19 Å contain two heterodimers per asymmetric unit and belong to the P3<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group. They were grown from 100 mM sodium formate and 20% polyethylene glycol 3350. The crystals grew in 4 days to a maximum final size of 0.45 × 0.25 × 0.025 mm and appear bipyramidal with a hexagonal base.

**Data Collection, Structure Solution, and Refinement**—The protein crystal was mounted from the mother liquor onto a cryoloop (Hampton Research) and then sequentially soaked in the reservoir solution containing an additional 5–25% glycerol in five steps and finally quickly frozen in liquid nitrogen. Diffraction data (λ = 0.9797 Å) were collected at 100 K using a MAR CCD detector at the French beamline for Investigation of Proteins (BM30A) at European Synchrotron Radiation Facility (ESRF) (Grenoble, France). The crystal diffracted beyond 2.6 Å resolution and was indexed with CrystAlisPro software (40). The crystal data collection statistics are given in Table I. The structure was solved by molecular replacement, using the MOLREP software from the CCP4 suite of programs (27). In an effort to gain new insights into the molecular mechanism controlling target gene exchange of corepressor and coactivator complexes is believed to underlie the molecular mechanism controlling target gene repression and activation (22, 23).

**Fluorescence Assays**—Fluorescence anisotropy assays were performed using a BEACON 2000 polarization instrument (Panvera Corp.) regulated at 4 °C, using filters for fluorescein at a peptide concentration of 2 μM. The buffer solution for assays was 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 10% (v/v) glycerol. All ligands (Table II) were present at concentrations above their respective K<sub>i</sub> values for binding to RAR and RXR and also at sufficient concentration to saturate both receptors. For the fluorescent peptide, due to the rapid establishment of the equilibrium, the measurements were initiated at the highest both concentrations of protein, and then the sample

**Table I**

| Crystallographic analysis |
|---------------------------|
| Parameter                  | Value     |
| Unit cell parameters (Å)   | a = b = 115.7, c = 247.2 |
| Solvent content (%)        | 65.5      |
| Resolution (Å) (last shell)| 30 – 2.9(3.06 – 2.9) |
| Number of observations     | 317,475   |
| Number of unique reflections | 42,989   |
| Redundancy                 | 7.4 (7.5)<sup>o</sup> |
| Completeness (%)           | 99.7 (99.6) |
| R<sub>i</sub> (%)          | 5.2 (2.0)  |
| R<sub>cryst</sub> (%)      | 25.35 (41.2) |
| R<sub>free</sub> (%)       | 29.64 (42.2) |
| r.m.s.d. bond lengths (Å)  | 0.014      |
| r.m.s.d. bond angles (°)   | 1.867      |
| Average B-factor (Å<sup>2</sup>) | 73.3     |
| Ramachandran (%)           | 83.0/140.6/40.6 |

<sup>o</sup> Values in parentheses refer to the highest resolution shell (3.06–2.9).

PAGE, purified using a Superdex 75 26/60 gel filtration column (Amersham Biosciences), the fractions containing the eluted heterodimer were pooled, and mixed with 2-fold molar excess of 9C-RA and 3-fold molar excess of TRAP220 NR box 2 peptide (641NHPMLMNLLKD-NPA<sup>654</sup>). The purified complex was concentrated to 15 mg/ml-1 and centrifuged for 30 min at 13,000 rpm prior to crystallization assays. Crystals with unit cell parameters a = b = 115.68 Å and c = 247.19 Å contain two heterodimers per asymmetric unit and belong to the P3<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group. They were grown from 100 mM sodium formate and 20% polyethylene glycol 3350. The crystals grew in 4 days to a maximum final size of 0.45 × 0.25 × 0.025 mm and appear bipyramidal with a hexagonal base.

**Data Collection, Structure Solution, and Refinement**—The protein crystal was mounted from the mother liquor onto a cryoloop (Hampton Research) and then sequentially soaked in the reservoir solution containing an additional 5–25% glycerol in five steps and finally quickly frozen in liquid nitrogen. Diffraction data (λ = 0.9797 Å) were collected at 100 K using a MAR CCD detector at the French beamline for Investigation of Proteins (BM30A) at European Synchrotron Radiation Facility (ESRF) (Grenoble, France). The crystal diffracted beyond 2.6 Å with useful data to 2.9 Å. Diffraction data were processed using MOSFLM (26) and scaled with SCALA from the CCP4 suite of programs (27). Data collection statistics are given in Table I. The structure was solved by molecular replacement, using the MOLREP software from the CCP4 package, with a truncated version of the RARβ/RXRα LBD heterodimer (28) as the search model. In this model, all water and ligand molecules were removed as well as the RARs and RXRα C-terminal helices H12 that were expected to adopt different conformations in the two dimers. The phases from the molecular replacement solution were refined with solvent flattening and 2-fold non-crystallographic symmetry averaging as implemented in the CCP4 dm program. The produced map was very clear for the RARα and the RXRα LBDs, the TRAP220 peptides, and the 9C-RA ligands. Multiple cycles of manual model building, including conversion of side chains from RARα to RARβ sequence, were carried out with the program O (29). Structure refinements were performed with CNS (30), using the maximum likelihood target and non-crystallographic symmetry constraints. The statistics of the structure refinement are summarized in Table I. The stereochemical quality of the model was assessed by use of the program PROCHECK (31). Fig. 1B was generated using SETOR (32). Figs. 1A, 2A, 4, and 5A were generated using PyMOL (33).

**Fluorescence Assays**—Fluorescence anisotropy assays were performed using a BEACON 2000 polarization instrument (Panvera Corp.) regulated at 4 °C, using filters for fluorescein at a peptide concentration of 2 μM. The buffer solution for assays was 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 10% (v/v) glycerol. All ligands (Table II) were present at concentrations above their respective K<sub>i</sub> values for binding to RAR and RXR and also at sufficient concentration to saturate both receptors. For the fluorescent peptide, due to the rapid establishment of the equilibrium, the measurements were initiated at the highest both concentrations of protein, and then the sample
was diluted successively by a factor of 0.75 with the buffer containing 2 nM of fluorescent peptide, allowing us to establish the titration curve. In contrast, for titration of the labeled SRC-1570–780 fragment, samples corresponding to each point of the titration curve were prepared in advance and incubated for 1 h. In all cases, for each titration point, anisotropy was measured successively until stabilized, and the reported values are the average of four measurements after stabilization. Binding data were analyzed using the package BIOEQS (34). Binding profiles were fit using a simple model assuming the stoichiometry of one SRC-1 by species (RAR/RXR heterodimer or RXR and RAR monomers). To determine affinities of other coactivator peptides for RAR and RXR, we used a strategy of competition. Prior to performing the assays, we estimated the ideal working concentrations of both protein partner and competitor peptide by simulating curves with BIOEQS. Finally, the fluorescent peptide was competed with increasing concentrations of unlabeled competitor peptide in the presence of a constant concentration of partner receptor. AM80 and CD3254 were used in the titrations as reference agonist ligands for RAR and RXR, respectively. All Kd values are reported in Tables III and IV.

Transactivation Assay—Cos1 cells plated in 24-well plates were transfected as described previously (35) using phosphate calcium precipitation. Ten nanograms of expression vectors pSG5-mRXRα wild type, F282A, F442A, and F443A were transfected together with 100 ng of DR1-tk-CAT reporter construct and 25 ng of CMV-β-galactosidase as internal control to normalize for transfection efficiency. The total quantity of DNA was adjusted to 1 μg. 15 h after transfection, cells were washed with phosphate-buffered saline and supplemented with fresh medium with or without 9C-RA for an additional 24 h. Intracellular levels of CAT were measured using a CAT enzyme-linked immunosorbent assay kit (Roche Applied Science) according to the manufacturer’s recommendations.

RESULTS AND DISCUSSION

Structure Determination—The purified heterodimer of E. coli-expressed LBDs of mouse RARβ2 (fragment 146–448, containing the entire D, E, and F regions) and mouse RXRα (fragment 227–467, containing the entire E region) was crystallized in the presence of 9C-RA and a synthetic peptide containing the second LXXLL motif (NR box 2) of the NR of the human TRAP220 coactivator (fragment 641–654). The structure was solved by molecular replacement using a truncated version of the previously determined RARα/RXRα heterodimer structure (see “Experimental Procedures”) as the search probe. Two solutions were obtained from the molecular replacement search with a correlation coefficient of 0.269 (next highest solution 0.189) and an R-factor of 0.575 consistent with two complexes in the asymmetric unit. The final solution comprising two heterodimers had a correlation coefficient of 0.472 (next highest solution 0.240) and an R-factor of 0.492. As the unliganded and H12-deleted heterodimer had been used for phasing, the very clear densities for the ligand in RARβ and RXRα LBDs and the continuous electron density observed for helix H12 of the two protomers confirmed the accuracy of the solution. The asymmetric unit contains two heterodimers, with each subunit binding one TRAP220 NR box 2 peptide and one ligand. Helix H2, the connection between H2 and H3 in RXRα, the D-region and the last 30 residues of the F-region in RARβ, could not be modeled due to poor electron density in these regions. The final model, refined to 2.9-Å resolution, comprises 462 protein and 21 peptide residues, 2 ligands, and 5 water molecules. The collection data and refinement statistics are summarized in Table I. The Rfree and Rcryst were 0.253 and 0.296, respectively, using all the data in the resolution range of 30–2.9 Å. PROCHECK analysis (31) showed that there were no residues in the disallowed regions of the Ramachandran plot. The geometry was very good, and the overall G factor was 0.17.

Overall Structure of the RARβ/RXRα Heterodimer—The RARβ and RXRα LBDs adopt the canonical three-layered α-helical sandwich structure seen in other nuclear receptor LBD structures with the ligands buried within the core of the proteins (17, 36, 37). A ribbon representation of the complex is shown in Fig. 1A. In contrast to the RARα/RXRα heterodimer (28), both protomers of the 9C-RA-bound RARβ/RXRα heterodimer adopt the active conformation with the “activation helix” H12 positioned against the protein, thereby essentially sealing the ligand-binding pockets (LBPs) of each protomer. This conformation generates a recognition surface constituted by mostly hydrophobic residues from helices H3, H4, and H12 of RARβ and RXRα, which allow one TRAP220 coactivator peptide to bind per protomer. The TRAP220 peptide folds as a two-turn amphipathic α-helix with the hydrophobic side chains packed against the agonist-induced RARβ and RXRα surfaces. In principle, the overall RARβ/RXRα heterodimeric arrangement closely resembles that of RARα/RXRα (28), peroxisome proliferator-activated receptor γ (PPARγ/RXRα) (38), and LXRα/RXRα (39) heterodimers with residues from helices H7, H9, H10, as well as loops L8–9 and L9–10 of each protomer forming the interface and a nearly two-fold axis relating the two subunits. The binding mode of 9C-RA to RXRα is identical to that observed previously (38, 40). Briefly, the ligand is completely buried within the ligand-binding pocket formed by residues located on helices H3, H5, H7, H11, and the β-turn (Fig. 1B, left panel). The majority of the contacts are van der Waals interactions except for the carboxylate group of the ligand that forms a salt bridge with the conserved Arg625 in helix H5 and a hydrogen bond with the backbone amide of Ala332 of the β-turn. Similarly, the binding mode of 9C-RA to RARα (Fig. 1B, right panel) closely resembles that already observed in RARγ (41) and involves the formation of a salt bridge between Arg269 of H5 and the carboxylate group, which in addition participates in a network of hydrogen bonds with Ser280 (β-turn) and a water molecule. The remaining residues of the LBP are hydrophobic and belong to helices H1, H3, H5, H11, H12, the connecting loop L6–7, and the β-turn. Electron density was observed for only 10 residues (out of 40) of the RARα C-terminal F region. These residues adopt an extended loop conformation projecting toward RXRα, establishing some interactions with helices H7 and H11 as well as loop L6–7 (Fig. 1A). In particular, Glu414 is engaged in two hydrogen bonds with the backbone amides of RXRα Ala340 and Ile350, whereas Pro412 makes van der Waals contacts with His440, Phe443, and Phe444 from H11 and Gly446 of L6–7 (not shown). However, the proximity of crystallographic or non-crystallographic symmetry-related
molecules stabilizing the C-terminal extension in the observed conformation strongly suggests that this RAR-RXR interaction is crystal packing-dependent. If this interaction would indeed occur in solution, it should stabilize the active conformation of RAR and therefore modify coregulator recruitment by RAR. However, no significant differences were observed for the binding affinities of fluorescent SRC-1 NR box 2 peptide to RAR with or without the F-region (Table IV).

The LXXLL-binding Site on RAR and RXR—The binding mode of the LXXLL motif to RAR and RXR LBDs is similar to that described in other LBD-coactivator peptide complex structures (38, 42, 43). Briefly, TRAP220 peptides are held in place through interactions of their leucine residues (Leu645, Leu648, and Leu649) with the hydrophobic groove generated by the C-terminal part of H3, the loop L3–4, H4, and H12 of RAR and RXR (Figs. 1A and 2A). In addition, each receptor shows two conserved residues that are hydrogen-bonded to a main-chain peptide bond of the LXXLL motif and generate a charge clamp that defines the precise length of the helical motif that can be docked to the cleft (not shown). These amino acids are a lysine at the C terminus of H3 (Lys237 and Lys284 in RAR and RXR, respectively) and a glutamate in H12 (Glu405 and Glu453 in RAR and RXR, respectively). Binding affinities of the interactions between the TRAP220 NR box 2 peptide and agonist-bound RAR and RXR are reported in Table III.

Interestingly, comparison of RXR holo-structures obtained in the absence or in the presence of coactivator NR box highlighted the important role played by three RXR phenylalanine residues during the peptide binding process. In the unbound receptor, Phe282 (H3), Phe442 (H11), and Phe455 (H12) are almost completely solvent exposed and make very little or no contact at all with other LBD residues (Fig. 2A). Upon NR box binding, we observed a concerted reorientation of the three
phenylalanine side chains that brings them in close contact to H12 and the peptide. Due to steric restrictions applied by Leu646 of the peptide, Phe492 rotates around the Cα-Cβ bond by 110° and forces Phe455 from H12 to pivot by 104°. A rigid-body translation of helix H12 toward H3 and the coactivator peptide of almost 2.0 Å allows Phe455 to be accommodated in a small, well suited hydrophobic pocket composed of residues from helices H3 (Phe282, Leu281, Leu285), H12 (Met459), and the peptide (Leu465, Met464, Leu468). Finally, Phe442 (H11) flips by 112° to contact Leu460 (H12) and forms together with Phe282 of H3 an “aromatic clamp” that locks H12 in the optimal conformation. Phe442 and Phe443 (H11) were previously found as being important for the stabilization of the unliganded form of RXR by partially filling the ligand-binding pocket of the apo-structure (44, 45). Single point mutations show that substitution of Phe443 with alanine has no effect on transcriptional activity of RXR homodimers in response to increasing concentrations of 9C-RA (Fig. 2B). In vivid contrast, disrupting the aromatic clamp by mutating Phe442 or Phe492 (H3) severely impairs maximal transactivation efficacy of 9C-RA-bound RXR homodimers, without modifying 9C-RA EC50. These structural and functional data demonstrate the importance of the aromatic clamp for formation and stabilization of the transcriptionally active RXR conformation and highlight the pivotal role of Phe442 in maintaining the integrity of both the apo- and the holoRXR structures.

**Ligand Binding and Coactivator Recruitment**—We used fluorescence anisotropy to quantify the ligand-dependent interaction between RXRα, RXRα, and SRC-1. We first determined the affinities of all three NR boxes of SRC-1 for RXRα and RXRα bound to their respective synthetic agonists AM80 and CD3254 (Table II). Fluorescein-labeled NR box 2 peptide was first titrated with either RXRα or RXRα, and its affinity was determined to be 0.47 (Kd) and 1.87 µM (Kd), respectively (Table IV). These complexes were then competed with unlabeled NR box 1, 2, and 3 peptides. As shown in Fig. 3A, the NR box 2 motif competes with itself and exhibits the highest affinity for RXRα and RXRα followed by NR box 3 and finally NR box 1, thereby confirming previous studies demonstrating that the presence of an intact NR box 2 in the NID of TIF-2 is sufficient for both efficient interaction with holoLBDs and stimulation of AF-2 activity, whereas NR boxes 1 and 3 are poorly efficient on their own (46).

To analyze the effect of ligands on the recruitment of coactivators by RXRα and RXRα, we measured the affinity of the interaction between monomeric receptors and either fluorescein-labeled NR box 2 peptide or the Alexa Fluor 488-labeled NID of the coactivator SRC-1 containing all three NR boxes (amino acid residues 570–780, SRC-1570–780). Titrations were carried out in the absence of ligand and in the presence of agonists (AM80 and CD3254 for RXRα and RXRα, respectively) or antagonists (BMS614 and UVI3003 for RXRα and RXRα, respectively) (Table II). Fig. 3B shows that apoRXRα binds SRC-1 fragments with higher affinity than does apoRXRα. In keeping with their agonistic properties, the binding of AM80 or CD3254 to their respective receptors strongly enhances the binding affinity of both the SRC-1 NR box 2 peptide and the SRC-1570–780 to RXRα and RXRα; note that RXRα-AM80 is slightly more efficient than RXRαCD3254 in recruiting the coactivator peptides. Interestingly, regardless of the receptor type (RXRα or RXRα), no significant difference in affinity is observed between the NR box 2 peptide and the full NID fragment, suggesting very similar binding modes. As expected, the binding of the antagonists BMS614 or UVI3003 to their specific receptors further reduces the binding affinity of SRC-1 NR box 2 to RXRα and RXRα when compared with the apo-receptors. Overall, it appears that the short 13-residue-peptide SRC-1 NR box 2 is sufficient to reproduce the essential aspects of the behavior of the entire SRC-1 NID fragment for monomeric receptor binding.

**Heterodimerization and Coactivator Recruitment**—Given the importance of heterodimerization in the retinoid signaling pathway, we addressed the question whether association of RXRα and RXRα in the context of the heterodimer and in response to a variety of ligands...
RAR/RXR Heterodimer and Coactivator Recruitment

An aromatic clamp stabilizes RXR holoH12. A, superposition of the structure of RXRa LBD (orange Ca tracing) bound to 9C-RA (Protein Data Bank ID code: 1FBY) onto the presently reported RXXa LBD (green Ca tracing) bound to 9C-RA and the TRAP220 peptide (green Ca tracing). The ligand as well as key residues discussed under “Results and Discussion” are depicted as thick lines. Red arrows indicate a concerted mechanism initiated by the binding of the LXXLL motif and ending with the formation of an aromatic clamp (involving Phe282, Phe442, and Phe455) that locks H12 in the active conformation. B, impaired transcriptional activity of the RXXF442A and RXXR282A mutants. Transcriptional activity of wild-type (wt) and mutant RXXa were analyzed by transient transactivation assays in Cos1 cells with the indicated RXXa (mutant) expression vectors and the DR1-tk-CAT reporter gene. Impairment of the transcriptional activity of RXXa by the F442A and F282A mutations underscores the importance of the aromatic clamp for holoH12 stabilization.

RARα/RXRα heterodimers liganded with the RXR-selective antagonist UVI3003 and the RAR-selective agonist AM80 (Fig. 3C; AM80/UVI3003) recruit SRC-1 NR box 2 and SRC-1570–780 with binding affinities almost identical to those observed for the monomeric RARα–AM80 complex (Fig. 3B), indicating that RXRa antagonist conformation does not affect the capacity of holoRARα to recruit coactivators. Likewise, SRC-1570–780 or its isolated NR box 2 displayed similar affinities for CD3254-ligated RXRa as monomer (Fig. 3B) or as heterodimer with antagonist-bound RARα (Fig. 3C; BMS614/CD3254). Together, these results demonstrate that, as far as a coactivator recruitment is concerned, RARα and RXRa act, within the heterodimer, independently from each other, like their monomers, which is in full agreement with the observation of only minimal structural alteration of the receptors upon dimerization. Indeed, except for some necessary side-chain reorientations at the dimerization interface, the structural superposition of the monomeric RARβ (47) and RXRa (40) LBDs onto their corresponding heterodimeric forms indicates very little variations between mono- and heterodimeric receptors. The root mean square deviations (r.m.s.d.) are 0.632 Å over 202 RARβ Ca atoms or 0.56 Å over 90 Ca atoms constitutive of the RARβ dimerization surface and 0.665 Å over 206 RXRa Ca atoms or 0.38 Å over 90 Ca atoms constitutive of the RXRa dimerization surface.

It has been previously observed that RAR and RXR ligands act synergistically (48) and that RAR/RXR heterodimers can cooperatively interact with the NR boxes of TIF2 (49). Indeed, heterodimers in which both RARs and RXRs are bound to selective agonists show enhanced binding affinity of SRC-1570–780 (Fig. 3C; compare AM80/CD3254 with the other ligand combinations), but the affinity of the isolated SRC-1 NR box 2 remains unchanged in the presence of these various ligands. This result underscores the above observation that heterodimerization does not modify the intrinsic capacity of individual receptors to interact with single LXXLL motifs and supports earlier observations suggesting that the synergy between receptor agonists could result from the formation of two binding sites on the heterodimer surface, which would interact with two LXXLL motifs of the same coactivator molecule (24, 43, 49). Furthermore, it appears that within the heterodimer, unliganded RAR and RXR contribute differently to the interaction with the coactivator. Although apoRAR is able to synergize with CD3254 (Fig. 3C; apo/CD3254) to strongly enhance the overall binding of SRC-1570–780 as compared with SRC-1 NR box 2 or to the heterodimer bound to the RAR antagonist BMS614 (BMS614/CD3254), the similar recruitment of SRC-1 NR box 2 and SRC-1570–780 by the AM80/apo or AM80/UVI3003 complexes clearly suggests that the unliganded RXR only marginally interacts with LXXLL motifs. Note in this respect that in contrast to cell-based assays in which serum-borne retinoic acid may complicate data analysis, we can exclude such interference in the present in vitro assays.

Having shown that dimerization does not significantly affect the structure of RAR and RXR LBDs or their binding affinity for isolated LXXLL motifs, we then addressed the question whether the dimerization interface is modified depending on the type of bound ligand, thereby providing a structural basis for the proposed allosteric communication between some heterodimeric receptors (50–52). The resolution of the structure of the agonist-bound RARβ/RXRα heterodimer allowed us to compare it with the previously determined crystal structure of the heterodimeric complex of RARα and RXRα LBDs in which the two subunits exhibit the antagonist conformation. The dimerization interface of the RARα-BMS614/RXRα-oleate complex (28) was superposed onto the 9C-RA-bound RARβ/RXRα heterodimer (Fig. 4). The Ca atoms of the dimerization surface (H7–H10) of the RXRα protomers were used to fix the superposition. This allowed us to compare the relative positions of the dimerization surfaces of the associated RAR protomers in each heterodimer. The r.m.s.d. for the 90 matched RXR Ca atoms within the H7–H10 region is 0.41
Å, indicating that the two RXR dimerization surfaces exhibit almost identical structures. The resulting r.m.s.d. between the dimerization surfaces of the RAR\(_\alpha\) and RAR\(\beta\) protomers also exhibits a low value (0.59 Å, over 86 C\(\alpha\)) (47), indicating that the two RAR/RXR heterodimer interfaces are very similar. A similar approach using the unliganded RXR\(\alpha\) homodimer structure (44) revealed an r.m.s.d. of 0.48 Å between the dimerization surfaces of apoRXR in the homodimer and holoRXR in the heterodimer (not shown). Thus, no major conformational differences could be observed for the dimerization surfaces of RAR and RXR in the absence or the presence of agonist or antagonist ligands. In keeping with the above structural analysis, the binding affinity of the RAR\(\alpha\)/AM80 subunit to SRC-1 NR box 2 remains unchanged irrespective of the presence or absence of RXR agonist or antagonist ligands (Fig. 3C; compare AM80/CD3254 with AM80/apo and AM80/UVI3003).

**Helix H11 Is a Sensor of Ligand Activity**—We compared the 9C-RA-bound RAR\(\beta\) holo-structure with the recently reported structure of RAR\(\beta\) bound to the synthetic agonist TTNPB (Table II) (47). Despite the overall similarity of the receptor conformations and the similar positioning of the ligands that bind with similar affinities to RAR\(\beta\), some differences with important functional implications can be observed. When compared with 9C-RA, TTNPB is much less flexible and binds to RAR\(\beta\) in a more elongated conformation (Fig. 5A). Moreover, TTNPB contains two additional methyl groups attached to the tetrahydronaphthalene ring that interact with Val\(^{388}\) and push helix H11 away from the pocket. In contrast, 9C-RA adopts a more bent conformation, allowing helix H11 to shift toward H12, therefore bringing Ala\(^{385}\) and Val\(^{388}\) too close to Leu\(^{407}\) from H12 (1.92 and 2.57 Å, respectively). As a consequence, helix H12 slightly translates along its helical axis toward helix H3,
and the side chains of Met₄₀⁶ and Leu₄₀⁷ undergo large conformational changes. Thus, the interaction surface with coactivators, of which helix H₁₂ is an integral part, is slightly different in the two complexes. Equilibrium binding of the fluorescein-labeled SRC-1 NR box 2 peptide to the RAR/H₉₂₅₂ LBD alone or bound to 9C-RA or TTNPB was analyzed to evaluate the impact of the structural differences described above on the recruitment of coactivators. As shown in Fig. 5B, although both TTNPB and 9C-RA can be considered as agonists because they facilitate interaction with the coactivator fragment, they show different efficiencies. Indeed, the SRC-1 NR box 2 fluorescent peptide is recruited to the RAR/H₉₂₅₂/TTNPB complex with a $K_d$ of 0.5 $\mu$M, whereas in the presence of 9C-RA, the affinity of the interaction is only 1.6 $\mu$M. Similarly, experiments conducted with RARβ also reveal 3-fold lower efficiency of 9C-RA-bound RARβ for recruiting SRC-1 NR box 2 when compared with RARα bound to TTNPB (Fig. 5C). In contrast, the RARα-specific agonist AM80 induces similar high affinity recruitment of SRC-1 NR box 2 when compared with TTNPB. Accordingly, both ligands share the important structural and chemical features discussed above. Therefore, it appears that because of suboptimal H₁₁-ligand interactions, 9C-RA is incapable of stabilizing helix H₁₂ in the conformation observed in the RAR/H₉₂₅₂/TTNPB complex structure, resulting in a less efficient recruitment of LXXLL motifs. Together, these data demonstrate how subtle chemical differences between various ligands can be sensed by helix H₁₁ and transferred to the activation helix through intimate packing contacts between helix H₁₁, loop L₁₁–₁₂, and H₁₂.

Concluding Remarks—In this study, we used x-ray crystallography and fluorescence anisotropy to get new insights into the ligand-dependent regulation of the interaction between RAR/RXR heterodimers and coactivators. Our data suggest a model of RAR/RXR heterodimer action in which the intrinsic capacity of each subunit to interact with LXXLL motifs of coactivators can be independently regulated by ligands without affecting the activity of the partner receptor. However, in full agreement with previous reports (49), we observed that the strength of the overall associ-
H11. Site-directed mutagenesis confirmed the functional importance of receptor antagonists (56, 57). Interestingly, the structure of helix H11 in the RARβ LBD complex with 9C-RA or TTNPB reveals that the position of helix H11 is during data collection. We thank Taru Deva for careful reading of the manuscript and the staff of the BM30A beamline at the ESRF in Grenoble, France.

11. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Kurokawa, R., Krones, A., Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A.,...