Retinoic Acid Receptor/Retinoid X Receptor Heterodimers Can Be Activated through Both Subunits Providing a Basis for Synergistic Transactivation and Cellular Differentiation*

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The receptor for 9-cis-retinoic acid, retinoid X receptor (RXR), forms heterodimers with several nuclear receptors, including the receptor for all-trans-retinoic acid, RAR. Previous studies have shown that retinoid acid receptor can be activated in RAR/RXR heterodimers, whereas RXR is believed to be a silent cofactor. In this report we show that efficient growth arrest and differentiation of the human monocytic cell line U-937 require activation of both RAR and RXR. Also, we demonstrate that the allosteric inhibition of RXR is not obligatory and that RXR can be activated in the RAR/RXR heterodimer in the presence of RAR ligands. Remarkably, RXR inhibition by RAR can also be relieved by an RAR antagonist. Moreover, the dose response of RXR agonists differ between RXR homodimers and RAR/RXR heterodimers, indicating that these complexes are pharmacologically distinct. Finally, the AF2 activation domain of both subunits contribute to activation even if only one of the receptors is associated with ligand. Our data emphasize the importance of signaling through both subunits of a heterodimer in the physiological response to retinoids and show that the activity of RXR is dependent on both the identity and the ligand binding state of its partner.

Vitamin A metabolites such as all-trans-(atRA)1 and 9-cis-retinoic acid (9cRA) play critical roles during embryonic development and adult physiology (1). At the cellular level retinoids influence processes such as growth and differentiation by specific effects on the regulation of gene expression. Both atRA and 9cRA bind to specific types of nuclear hormone receptors that belong to a large family of conserved proteins, including receptors for steroid hormones, thyroid hormone, and vitamin D₃ (2). In addition, a large number of related proteins have been identified which lack known ligands and are therefore referred to as orphan receptors (3).

Nuclear hormone receptors act as ligand inducible transcription factors and bind to specific DNA binding sites, termed hormone response elements, in promoters of regulated genes. The receptors contain distinct functional domains, including a well conserved DNA binding domain and a somewhat less conserved C-terminal ligand binding domain (LBD). Recent studies have revealed that ligand binding mediates a structural transition, which involves repositioning of a α-helix in the core of the C-terminal activation domain (AF2) (4–9). In activated receptors the AF2 domain binds to accessory proteins named co-activators which in turn are believed to mediate contacts with the basal transcriptional machinery and thereby trigger transcriptional activation (10–13). In several cases receptors have been shown to function as repressors of transcription, an activity that is believed to be mediated by a distinct type of accessory proteins termed co-repressors (14–17).

Two different types of retinoid receptors have been identified. RAR is activated by both atRA and 9cRA, whereas RXR is activated only by 9cRA (1). RXR has been shown to regulate gene expression in response to 9cRA both as homodimers and in heterodimeric complexes with orphan receptors such as LXR, NGF1-B, and FXR (18). In addition to its function as a 9cRA-inducible receptor, RXR plays a central role as a heterodimerizing co-factor in gene regulatory events mediated by other members of the nuclear receptor super family, i.e. RAR, the thyroid hormone receptor (TR) and the vitamin D₃ receptor (VDR) (3). In these heterodimers RXR has been shown to be allosterically blocked and function as a silent co-receptor (19–21).

In contrast, previous studies have demonstrated that both RAR and RXR contribute to the activation of certain retinoid acid-responsive elements (RAREs) (6). Also, several cell lines which respond to retinoids require activation of both RAR and RXR pathways for efficient induction of cellular processes, e.g. growth arrest of cervical carcinoma cells (22), differentiation of embryonic carcinoma cells (23), and granulocytic differentiation of HL-60 cells (24). These results either suggest that two distinct pathways synergize in mediating the biological response of retinoids or, alternatively, in contrast to the concept of RXR as a silent heterodimerization partner of RAR, point to the possibility that both subunits of the RAR/RXR heterodimer can be activated by ligand.

In this study we have used synthetic RAR- and RXR-selective ligands to demonstrate that the monoblastic cell line U-937 requires activation of both RAR and RXR for efficient induction of the monocytic differentiation program. To analyze the molecular mechanism behind this cooperativity between RAR and RXR, we utilized the GAL4 system, which enables us to study the retinoid response through RAR/RXR heterodimers independent of endogenous receptor complexes that bind to RA response elements. Although RXR can be shown to be in a repressed state in complex with RAR, we show that this inhibition of RXR is relieved by RAR ligand binding. Even binding...
of an RAR antagonist allows activation of the RXR moiety in the RAR/RXR heterodimer. Moreover, we demonstrate that the RXR AF2 domain is required for efficient activation of ligands binding to RAR, TR, VDR, and PPAR also in the absence of RXR ligand. In conclusion, our data demonstrate that RXR is subject to both positive and negative allosteric regulation by its heterodimeric partner that results in repression, partial activation, or full activation of RXR depending on the availability of ligands for either or both subunits.

EXPERIMENTAL PROCEDURES

**Induction of U-937 Cell Differentiation—** U-937 cells (25) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc., Uxbridge, United Kingdom) and antibiotics (100 units/ml of penicillin and 50 μg/ml of streptomycin). Cells at a concentration of 1 × 10⁶/ml were exposed to different retinoid compounds for 4 days. Retinoids used were atRA (Sigma), 9cRA (Hoffmann-LaRoche, Basel, Switzerland), and TTNPB and SR11237 (19). Differentiation antigen expression was analyzed by flow immunocytometry as described (26). Primary antibodies used were LeuM5 (CD11c; Becton Dickinson, Mountain View, CA), GoH3 (CD49f; Immunotech, Marseille, France), and YTH71.3 (CD66a; kindly provided by Prof. H. Waldman, Department of Pathology, University of Cambridge, Cambridge, United Kingdom). Fluorescein isothiocyanate-labeled goat-anti-mouse Fab(′)₂ fragments (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands) were used as a secondary reagent for CD11c-labeled cells and fluorescein isothiocyanate-labeled rabbit-anti-rat Fab(′)₂ fragments for CD66a- and CD49f-labeled cells. Cell cycle analysis was performed as described (27). The data were processed with the MacCycle software (Phoenix Flow Systems, San Diego, CA) to obtain DNA distribution histograms as shown in Fig. 1C. The cellular morphology was examined by light microscopy after cytosin centrifugation and staining with May-Grünewald-Giemsa solution.

**Plasmids—** The luciferase reporters used in transfection experiments contain the promoter and enhancer sequences of the hRARα gene promoter RARE (βRE3-4tk-luc) or four copies of the GAL4 binding site (MH100×4tk-luc) cloned upstream of the herpes simplex virus thymidine kinase gene minimal promoter (28). Receptors and derivatives of receptors were expressed from pCMX expression vectors containing the cytomegalovirus promoter and enhancer sequences (29). pCMX-GAL4-RAR and pCMX-GAL4-RXR contain the sequences encoding the ligand binding domains of hRARα (from Glu-185) and hRXRα (from Glu-203), cloned in frame after a sequence encoding the 78 amino acids from the trans-activation domain encoded in pVP16C1 (Novagen, Madison, WI) (28). Each well was transfected with 100 ng of reporter plasmid, 100 ng of receptor expression vector, and 200 ng of pCMX-βgal reference plasmid containing the β-galactosidase gene. Additions to each well were adjusted to contain a constant amount of DNA and of pCMX expression vectors for 4 days. Twenty million U-937 cells were harvested, washed, and suspended in a cuvette of 0.5 ml of RPMI 1640 containing 20 μg of βRE3-4tk-luc plasmid and 20 μg of RSV-βgal vector. The cells were transfected by electroporation at 0.30 kV/960 microfarads (Gene Pulser, Bio-Rad), left on ice for 10 min, and then transferred to culture medium. Multiple transfections of cells were performed and all were performed in the incubator for 1–2 h. Then the cells were split and stimulated over night in triplicate with retinoids as indicated. Cell extracts were prepared by three cycles of freezing and thawing in 0.1 M potassium phosphate (pH 7.4) and assayed for luciferase and β-galactosidase activity (luminescent β-galactosidase detection kit, Clontech, Palo Alto, CA) in a luminometer (Lumat LB9501, Berthold, Bad Wildbad, Germany). Variance between data points within each experiment were less than 20%. All luciferase activities were normalized to β-galactosidase activity.

RESULTS

RAR and RXR Synergize in the Differentiation of U-937 Cells—The human U-937 cell line is arrested at a monoblastic stage of hematopoietic development and differentiates along the monocytic lineage upon atRA treatment (31). During the differentiation process the cells up-regulate surface antigens associated with the terminally differentiated phenotype and acquire a monocyte-like morphology. High concentrations of atRA (in the micromolar range) in cell cultures results in isomerization to 9cRA, which can activate both types of retinoid receptors. Therefore, to analyze the individual contribution of RAR and RXR activation for the differentiation process, we stimulated U-937 cells with TTNPB and SR11237, synthetic retinoids that specifically bind and activate RAR and RXR, respectively (19, 32). After 4 days of 9cRA stimulation over 90% of the U-937 cells express the surface antigens CD11c, CD49f, and CD66a, which are characteristic markers of the differentiated phenotype (Fig. 1A) (26). In contrast, TTNPB and SR11237 are by themselves poor inducers of antigen expression. However, when administered together, TTNPB and SR11237 induce differentiation antigens as efficiently as 9cRA (Fig. 1A).

RA-differentiated U-937 cells develop a characteristic morphologic with lobulated nuclei as shown in Fig. 1B. Cells stimulated for 8 days with either TTNPB or SR11237 retained the blast-like phenotype of nondifferentiated cells. However, co-stimulation with TTNPB and SR11237 resulted in the typical morphology of mature atRA-differentiated cells (TT+SR; Fig. 1B). Similarly, TTNPB and SR11237 synergize in the induction of growth arrest in the G1/G2 phase of the cell cycle, consistent with the tight coupling between cessation of proliferation and terminal differentiation in these cells (Fig. 1C). Flow cytometric analysis of propidium iodide-stained cell nuclei prepared from cells treated with either TTNPB or SR11237 showed a slightly larger fraction of the cells in the G1/G2 peak, compared with unstimulated exponentially growing cells, indicating some inhibition of proliferation. However, a complete cell cycle arrest in the G1/G2 phase, similar to that observed for atRA, required co-stimulation with TTNPB and SR11237. Thus, the activation of both RAR and RXR is critical for the efficient induction of the hallmarks of RA induced differentiation, i.e. growth arrest in G1/G2, up-regulation of differentiation antigens, and development of the distinct morphology of mature monocytes.

We next wished to examine if the effect of combined RAR and RXR stimulation on U-937 cell differentiation could result from synergistic in transactivation of an RARE. Transient transfections of U-937 cells were performed with a reporter construct containing the RARE from the RARα promoter (βRE3) (33, 34). 9cRA is a strong inducer of βRE reporter activity through the endogenous receptors of U-937 cells (35) (Fig. 2). TTNPB stimulation resulted in a moderate activation while SR11237 only induced a minor response. Co-stimulation with TTNPB and SR11237 potently activated the reporter. This effect was more
Synergistic Activation of RAR/RXR Heterodimers

Both Subunits in RAR/RXR Heterodimers Can Be Activated when Receptors Are Simultaneously Exposed to RAR and RXR Ligands—To investigate the possibility that both RAR and RXR activation is required for U-937 differentiation, we utilized GAL4 receptor hybrids and a luciferase reporter containing GAL4 binding sites in its promoter (Fig. 3A). A two-amino acid substitution was introduced in the RAR activation domain (AF2) that would eliminate activation through the RAR subunit without affecting its ability to bind ligand. This assumption relies on previous mutations introduced in RAR in which activation, but not ligand binding, is abolished (6). The GAL4 hybrid receptor containing the ligand binding domain of such an RAR derivative (GAL4-RARmAF2; Fig. 3A) can heterodimerize with a VP16-fused RXR as efficiently as GAL4-RAR as demonstrated in the two-hybrid transfection experiment performed in human JEG-3 cells (Fig. 3B). VP16-fused RXR alone did not activate the reporter (data not shown). Importantly, the data in Fig. 3C demonstrate that heterodimers formed between the GAL4-RARmAF2 and RXR can be activated by SR11237 in the presence but not in the absence of the RAR agonist TTPNB. Similarly, a low dose of 9cRA also allows such activation through the RXR subunit of the GAL4-RARmAF2/RXR heterodimer. Notably, activation by RXR is weak when heterodimerizing with GAL4-RARmAF2, indicating that the AF2 mutation affects the efficiency by which RXR can be activated (see below). The data corroborate previous observations showing that RXR is allosterically blocked in complex with RAR, but clearly demonstrate that inhibition can be relieved by binding of an RAR agonist to the RAR subunit of the heterodimer.

An RAR Antagonist Can Relieve the Allosteric Block of RXR—We next wished to test if also an RAR antagonist could lead to a conformational shift that would allow RXR to be activated in an RAR/RXR heterodimer. Therefore, transfected JEG-3 cells were treated with SR11237 and an RAR antagonist, Ro41-5253 (36), which binds to RAR but does not lead to its activation (Fig. 4A). Interestingly, heterodimers formed between GAL4-RAR and RXR respond weakly to SR11237 in the presence, but not in the absence, of Ro41-5253, demonstrating that RXR inhibition is partly relieved under these conditions (Fig. 4B).RXR subunits can be activated in RAR/RXR heterodimers, we wished to assay RXR activation independently of RAR. Therefore, we analyzed the pharmacological properties of the RAR/RXR heterodimers in RAR- and RXR-responsive systems.

RXR/RXR Homodimers and RAR/RXR Heterodimers Are Pharmacologically Distinct in Their Response to Synthetic RXR Ligands—Our results together with previously published data clearly show that RAR can allosterically influence RXR. Therefore, we analyzed the pharmacological properties of the RAR-
RXR complex in comparison with RXR/RXR homodimers in response to different RXR activators. In these experiments dose-response curves using different RXR activators were determined for activation by GAL-RXR or GAL-RARmAF2/RXR heterodimers (Fig. 5A). Cells expressing either GAL-RXR or the combination of GAL-RARmAF2 and RXR were treated with increasing amounts of the different RXR activators. Moreover, GAL-RARmAF2- and RXR-transfected cells were simultaneously incubated with a constant amount of TTNPB, since a ligand for RAR is a prerequisite for RXR activation as demonstrated above. Activation by three compounds, SR11237, LG153, and atRA, showed similar dose-response curves when comparing GAL4-RXR (homodimer) and GAL4-RARmAF2/RXR (heterodimer) activation (Fig. 5A). Similar results were seen with RXR activators such as 9cRA and methoprene acid (37) (data not shown). Interestingly, as demonstrated in Fig. 5B, one of the tested ligands, SR11234 (19), was a weak activator of the GAL4-RARmAF2/RXR heterodimer, whereas it potently activated GAL4-RXR. These results demonstrate that the two complexes are pharmacologically distinct and also suggest that RXR homodimers and RAR/RXR heterodimers respond differently to RXR ligands.

The AF2 Domain of Both Subunits Contributes to Activation of RXR Heterodimeric Complexes—Notably, RXR is only weakly activated in complex with RAR in the experiments displayed in Figs. 3C and 4B. In these experiments the AF2 of RAR is inactive, either because the RAR AF2 is mutated (Fig. 3C) or an RAR antagonist is used (Fig. 4B). Thus, the data indicate that efficient activation is dependent on the functional integrity of both AF2 domains of a heterodimeric complex. To test if the activation of RAR, in the context of an RAR/RXR heterodimer, also depended on two AF2 domains, a two-amino acid substitution was introduced in RXR AF2 (Fig. 6A). This RXR derivative (GAL4-RXRmAF2) is inactive when transfected cells are treated with SR11237 but heterodimerizes efficiently with RAR, as indicated by the two-hybrid interaction between GAL4-RXRmAF2 and a VP16-RAR derivative (data not shown). Indeed, a heterodimer formed between GAL4-RXRmAF2 and RAR is only weakly active in the presence of TTNPB, demonstrating that the AF2 function of RXR is required, although TTNPB only binds to the RAR subunit of the complex.
In addition, other heterodimeric partners, including TR, VDR, and PPARγ, also require an intact RXR AF2 domain, since activation is diminished when these receptors are co-expressed with GAL4-RXRmAF2 and treated with triiodothyronine, vitamin D3, and the specific PPARγ ligand BRL49653 (38), respectively (Fig. 6B). Consistent with this result, a truncated derivative of RXR (RXR443), which lacks an intact AF2 domain, acts as a dominant negative inhibitor of RAR activation from a reporter containing the βRE (Fig. 6C). Together, these results demonstrate that an intact RXR AF2 is required for efficient ligand activation of several RXR partners.

**DISCUSSION**

RXR participates in several signaling pathways by forming heterodimers with receptors such as RAR, TR, and VDR. In such heterodimers; however, RXR has been suggested to function as a silent partner, which promotes high affinity DNA binding of the ligand binding subunit to its specific hormone
response this notion we show in this report that RXR ligands have little effect on U-937 cells. Still, some reports favor the idea that both subunits in an RXR heterodimer can be activated by ligand, as supported by cooperation between RAR and RXR ligands in RARE activation and induction of RA-induced genes (6, 23). Consistent with this concept our results demonstrate that both subunits in the RAR/RXR heterodimer can indeed be activated when both RAR and RXR ligands are present simultaneously. This also appears to be a prerequisite for the biological response, i.e. differentiation of U-937 cells.

The combined use of the GAL4 system and the RAR AF2 mutant demonstrated how RAR and RXR ligands affected RXR activation in a context where the luciferase reporter gene was not affected by ligand dependent trans-activation of RAR. Also, this experimental approach ensured that transcriptional influence from various endogenous receptor complexes was avoided. The results show that RXR is responsive to selective RXR ligands in the presence, but not in the absence, of RAR ligands. Previously published experiments demonstrated that RXR is allosterically inhibited in complex with RAR, which could be mechanistically explained by RXRs inability to bind ligand in RAR/RXR heterodimers (20, 21). Our experiments confirm this view but, in addition, suggest that RXR ligand induces a conformational change which not only affects the ligand binding subunit, RAR, but is also translated by RXR to relieve inhibition of ligand binding. Previous biochemical experiments support this view and have suggested that both subunits of RXR/RXR heterodimers can indeed associate with ligand (21, 24).

Surprisingly, even an RAR antagonist combined with RXR ligands allow RXR activation. It was recently shown that a combination of an RAR antagonist and an RXR agonist can induce differentiation and apoptosis of promyelocytic NB4 cells, which express the PML/RARα fusion oncprotein (39). Our experiments demonstrate that cooperation between RAR antagonists and RXR agonists in these cells may be explained by allosteric receptor interactions within RAR/RXR heterodimer complexes.

Using several synthetic RXR ligands we could show that the RAR/RXR heterodimer is pharmacologically distinct from RAR/RXR homodimers. The experiments demonstrated that the synthetic RXR ligand SR11234, as opposed to other tested RXR ligands, is a weak activator of RAR/RXR heterodimers. Since a low level of activation could be observed (Fig. 5; data not shown) we conclude that SR11234 can associate with RAR/RXR heterodimers and that the deficiency resides in an inability to promote efficient activation rather than ligand binding to the RAR/RXR heterodimer. SR11234 and SR11237 are structurally similar benzoic acid derivatives with ketal and thioketal substituent groups, respectively (19). Thus, we speculate that the ketal as opposed to the thiketal group may interfere with the efficiency by which co-activators and/or co-repressors assemble with RAR/RXR heterodimers but not RAR/RXR homodimers. Such differences in factor assembly have been demonstrated recently comparing the activities of novel synthetic RXR ligands with opposite effects on RAR/RXR heterodimers (40).

RA has been used in treatment of a number of different cancers, such as promyelocytic leukemia, head and neck carcinoma, and skin cancer (41). Despite these interesting clinical developments, remarkably little is known as to how RA acts to suppress cancer cell growth. Previously published data have established that certain RAR antagonists, termed “dissociated retinoids,” are functional in their ability to inhibit AP1 activity, although they fail to trigger transcriptional activation by RAR (42, 43). These retinoids specifically inhibited the growth of lung and breast cancer cells, whereas growth suppression of other cell types required “classical” activation of retinoid receptors by atRA (42). In addition, several naturally occurring retinoids exist, some of which are potent activators of RAR (44–46). It is plausible that also naturally occurring compounds, similar to SR11234, will eventually turn out to selectively affect distinct RXR complexes. Therefore highly selective cellular responses could be achieved with different combinations of synthetic and natural retinoids. Clinically, it can be anticipated that combinations of pharmacologically distinct retinoids could increase both the specificity and the potency of retinoid action in cancer therapy.

The emerging knowledge on co-activators/repressors and the determination of the crystal structure of nuclear receptor ligand binding domains may provide clues as to the mechanism behind the versatile allosteric control of RXR reported in this paper. Although the structure for one and the same receptor has yet to be shown both in a ligand-bound and unbound state, several important conclusions can be made on the nature of the conformational change occurring in liganded receptors (7–9). Comparison of the apo-LBD of RXRs with the holo-LBDs of RARy and TRα suggests that a ligand-induced conformational shift creates a more compact LBD fold (47). Furthermore, the protruding AF2 core α-helix, encompassing helix 12 in the described nuclear receptor LBD structure, folds back against the LBD, where it will be in close contact with the bound ligand. Thus, ligand induces both local and global conformational changes that easily could be envisioned to affect a heterodimerization partner. Also, several studies have demonstrated the importance of the AF2 core for association with co-activators in the presence of ligand (10–13). Unexpectedly, our results demonstrated that the integrity of the AF2 domain of both subunits in RXR heterodimers are important for efficient activation, even when only one subunit of the heterodimer is activated by ligand. Experiments with an RXR AF2 mutant emphasized the importance of two AF2 domains in heterodimers such as RAR/RXR, TR/RXR, VDR/RXR, and PPAR/RXR, in which ligand-induced transcription depended on the AF2 domain of the nonliganded partner RXR. Conceivably, ligand binding to the heterodimerization partner could promote a conformational shift, which also affects the RXR AF2 and induces a conformation that resembles the “activated” state. Alternatively, co-activators may associate weakly with RXR AF2 domains also in the absence of RXR ligands. In a heterodimer with only one liganded receptor, synergistic co-activator binding may result from a combined weak and strong association with the nonliganded and liganded receptor partners, respectively.

In conclusion, ligand binding to RAR has two important influences on RAR/RXR heterodimers. First, RAR ligand binding results in a partial transcriptional activation that is dependent on the RXR AF2. Second, an allosteric change is induced that allows RXR to bind ligand. Furthermore, in the presence of both ligands, heterodimers mediate synergistic transcriptional activation. Our data help to explain why synergistic physiological responses to both RAR and RXR are observed in many cell types such as U-937 cells. Also, a common and consistent feature of most, if not all, mammalian cells is the combined expression of RAR and RXR, suggesting a critical and central role for RAR/RXR heterodimers in responses to retinoids. The results emphasize the importance of observing each receptor in a heterodimer as an integrated component in a complex in which the receptors are regulated by mutual interactions involving the transactivation and ligand binding domains of both receptors.

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Retinoic Acid Receptor/Retinoid X Receptor Heterodimers Can Be Activated through Both Subunits Providing a Basis for Synergistic Transactivation and Cellular Differentiation

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