Differential 9-cis-Retinoic Acid-dependent Transcriptional Activation by Murine Retinoid X Receptor α (RXRα) and RXRβ

ROLE OF CELL TYPE AND RXR DOMAINS*

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The 9-cis-retinoic acid (9cRA)-inducible enhancer of the rat cellular retinol-binding protein type II gene (CRBP II) was shown to be differentially regulated by the murine retinoid X receptor α (RXRα) as compared with RXRβ. Transient transfection assays performed in NIH 3T3 fibroblast cells demonstrated that RXRα yielded a high level of 9cRA-dependent transcription of a reporter gene linked to the CRBP II enhancer, when compared with RXRβ. This effect was cell type-dependent, since both receptors elicited comparable transcriptional activation of the same reporter in P19 embryonal carcinoma cells. To further explore the structural determinants responsible for the differences between these two receptors, a series of chimeric receptor constructs were made. Co-transfection assays utilizing these chimeras demonstrated that both the N terminus and the hinge region connecting the DNA binding domain with the ligand binding domain of RXRα were responsible for the high level of 9cRA-dependent transcription observed in NIH 3T3 cells. Furthermore, the hinge region of RXRα was shown to be necessary to repress, in the absence of hormone, the transcriptional activation function located in the N-terminal domain of RXRα. These results stress the importance of functional links between different RXR domains and suggest an RXR subtype and cell type-dependent specificity in the control of the 9cRA response.

The molecular mechanisms by which members of the steroid and thyroid hormone receptors superfamily regulate the expression of target genes are still unclear. RXRs, in particular, exert pleiotropic functions due to their ability to heterodimerize with a variety of other receptors within this family, including retinoic acid receptors, thyroid hormone, and vitamin D receptors. Thus, RXRs are assumed to play a central role in modulating the cellular response to multiple signaling pathways (1–7). In addition, RXR homodimers can transactivate RXR-responsive elements (RXREs) such as that found within the rat CRBP II gene (8–10).

A further element of complexity is added by the presence of multiple subtypes of the receptors (α, β, and γ) that show dissimilar transcriptional activities on the same response element (11). In addition, murine RXR receptor subtypes have been shown to be differentially expressed during development (see Refs. 12–14 for reviews). However, the structural features involved in subtype-specific gene regulation are unknown.

Nuclear hormone receptors have a modular structure that consists of at least five regions (denoted A–E in Fig. 2; for reviews see Refs. 13–15). The most evolutionary conserved regions are the DNA binding domain (DBD, region C) and the ligand binding domain (region E). The C region directly contacts their cognate response elements, while the E domain is responsible for ligand binding, transcriptional activation function (AF2), and homo- and heterodimerization (1–7, 13–15, 19). The D domain (hinge region) exhibits much less sequence similarity among receptor subtypes and has recently been shown to play a role in the specificity and polarity of binding of receptor heterodimers to DNA response elements (14–18, 24, 25). The N-terminal or A/B domain appears to be partially responsible for specific differences among retinoic acid receptor subtypes (11, 19). This region contains a transactivation domain function (AF-1), which can synergize with AF-2, located in the ligand binding domain (reviewed in Ref. 14).

In this report we demonstrate that RXRα and RXRβ differentially transactivate the RXRE from the rat CRBP II promoter in NIH 3T3 fibroblast cells but similarly in P19 embryonal carcinoma cells. We have tested a series of chimeric receptors to elucidate the domains responsible for the differences in transcriptional activation. Our results showed that both the A/B and D domains are involved in dictating the output of the cell-dependent transcriptional response. Hence, these two regions cooperate with each other and potentially with other cell-specific factors to maintain the specificity of the response, thus providing the functional elements necessary for eliciting subtype-specific and cell type-specific transcriptional regulation.

EXPERIMENTAL PROCEDURES

Plasmids—The reporter construct CRBPII(2)k-luc, containing two copies of the CRBP II RXRE in front of the thymidine kinase promoter driving the luciferase coding sequence, was described previously (9). RSVE-2RIIBP (7), expressing the mouse RXRβ, was utilized to generate the chimeras as described above. The RXRα clone was a generous gift from Drs. Ron Evans and David Mangelsdorf (12).

Construction of Chimeras—Five-part chimeric receptors were created from the original cDNAs coding for RXRα and RXRβ (7, 12). The sites used were either existing restriction sites or sites created by PCR introducing silent mutations. All sites and regions were amplified by

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1 The abbreviations used are: RXR, retinoid X receptor; RXRE, RXR-responsive element; CRBP II, cellular retinol-binding protein type II; DBD, DNA binding domain; PCR, polymerase chain reaction; bp, base pair.
PCRs using Pfu polymerase (Stratagene). Each chimeric exchange was made in regions of high conservation, and no novel amino acids were introduced. PCR fragments were first cloned into pCRscript (Stratagene) and sequenced utilizing an ABI DNA sequencer. Subcloning was then performed in pSP72 to characterize the constructs by in vitro transcription-translation (6). Following confirmation, the full-length chimeric genes were cleaved from the pSP72 construct by digestion with HindIII/HpaI and cloned into the eukaryotic expression vector RSVneo cleaved with HindIII/HpaI, resulting in replacement of the neo sequence with that of the receptors. When it was necessary to do into the HindIII site, the HpaI site in pSP72 was eliminated by insertion of an oligonucleotide containing Smal, DraI, and SnaB1 restriction sites, and one of them was utilized to do in the HpaI site of RSVneo (6). For the RXRα receptor the restriction sites used to make the five-part chimeras were HindII site (artificially created) at base pair (bp) 85 of the original DNA, a PvuI site at bp 560, a HindIII site at bp 738, an Xbal site at bp 818, a BamHI site at bp 964, and a HpaI site at bp 1532. The chimeric parts were named a1, a2, n3, a4, and 5 (see Fig. 2). The correct PvuI site for RXRα was selected by partial digestion. RXRα/a1[a3a4a5] was made by deleting the region between the HindIII site and PvuI site of the RXRα construct and replacing it with the AUG start codon and 12 nucleotides upstream from position 315 of RXRα. For the RXRβ receptor, the corresponding sites used were the HindIII site created by PCR immediately upstream of the native Ncol site at bp 155, a PvuI site at bp 400, a HindIII site at bp 578, an Xbal site at bp 652, a BamHI site at bp 808, and the HpaI site at bp 1360. The chimeric parts were named β1, β2, β3, β4, and β5 (see Fig. 2).

Transfections—Transfections into NIH 3T3 cells were performed as described (20). Briefly, for NIH 3T3 cells 10 μg of reporter plasmid was electroporated into 4 × 10⁶ cells with 12.5 μg of native or chimeric receptor plasmid. Following the transfections, the cells were cultured for 60 h with 1 μM 9cRA (a gift from Dr. Arthur Levin at Hoffmann-La Roche). P19 cells were transfected by using LipofectAMINE (LifeTechnologies, Inc.) as described (22). Following an overnight incubation with the DNA/LipofectAMINE mixture, 9cRA (1 μM) was added, and the cells were incubated for an additional 24 h in the presence of the hormone. After lysing the cells, luciferase activity assays were performed as described (23) and normalized by utilizing a Cytomegalovirus β-galactosidase plasmid included in the transfection. Results represent the average of at least three independent transfections performed in duplicate.

**RESULTS AND DISCUSSION**

Several reports have suggested that there may be RXR subtype-specific differences in 9cRA-dependent transcriptional activation on particular response elements (11, 12, 19). We compared the transcriptional activation mediated by RXRα and RXRβ in transient transfection assays in NIH 3T3 cells cotransfected with either receptor and a reporter construct containing the luciferase reporter gene linked to the CRBP II response element than RXRβ. Western immunoblot analysis performed after transfection of RXRα and RXRβ constructs containing C termini tagged with flag epitope demonstrated that the difference in transcriptional activation could not be attributed simply to differences in the levels of expression of these receptors (data not shown). We also examined the transcriptional activation in P19 cells. Interestingly, both RXRα and RXRβ showed very similar levels of transcriptional activation in P19 cells (Fig. 1).

In order to better understand the structural requirements underlying the differences in transcriptional activation observed in NIH 3T3 cells, we constructed a series of chimeras between these two receptors (Fig. 2). Each receptor was subdivided into five parts. Parts 1, 2, 3, 4, and 5 correspond to the A/B, C, N and C termini of D, and the E domain, respectively. The expected molecular masses for the chimeric receptors were calculated using the molecular masses of individual parts (data not shown). Finally, the functional integrity of each receptor was confirmed by transfection of P19 embryonal carcinoma cells. Each chimera in P19 cells showed comparable levels of transcriptional activation, differing less than 2-fold, in the presence of 9cRA on the CRBP II response element (Fig. 3).

Next, we tested each chimera in NIH 3T3 cells to identify the functional determinants responsible for the differences between the two receptors (Fig. 4). As shown in Fig. 1, RXRα was approximately 20-fold more active than RXRβ under identical conditions of transfection (compare lane 1 with 2 in Fig. 4A). This difference was independent of part 2 (the C domain) since the exchange of the RXRα part 2 with the corresponding part 2 of RXRβ (RXRα/a1[a3a4a5] and RXRβ/a1[a3a4a5]) was as efficient as the RXRα (compare lane 1 with 15 in Fig. 4A). Similarly, the RXRβ part 2 substituted for the RXRα part 2 in the context of the RXRβ receptor (RXRβ/a1[a3a4a5]) (compare lane 2 with 9). This result was expected, since the two receptors are more than 95% homologous in the C domain corresponding to DBD (Fig. 2A). However, it was not possible to completely rule out any involvement of part 2, since RXRβ/a1[a3a4a5] gave lower transactivation than RXRβ/a1[a3a4a5] (compare lane 13 with 14), suggesting that the C domain may contribute to optimal folding.

In agreement with a previous report (23), our results point out that the A/B domain of RXRα is absolutely required for achieving the high level of transcriptional activation observed with RXRα/a1[a3a4a5], since, when partially deleted (RXRα/a1[a3a4]), a 4-fold decrease in transcriptional activation was observed (Fig. 4A, compare lane 1 with 3). In addition, the A/B domain (part 1) of RXRβ could not functionally substitute for that of RXRα (RXRβ/a1[a3a4a5]) (compare lane 1 with 13). These results confirm an earlier report (19) suggesting that the A/B domain of RXRα, but not RXRβ, exhibits a transcriptional activating function (AF-1).

The A/B domain of RXRα, however, was not sufficient to induce the high level of 9cRA-dependent reporter activation observed with RXRα/a1[a3a4], when linked to the C terminus (D and E domains, parts 3, 4, 5) of RXRβ (RXRβ/a1[a3a5]) (Fig. 4A, compare lane 6 with 1). In addition, the C terminus of RXRα mediated slightly elevated transcription above the levels found for RXRβ, when linked to the RXRβ A/B domain (RXRβ/a1[a3a4a5]) (compare lane 2 with 14). Taken together, these results imply that both the A/B domain and the C terminus of RXRα are necessary for high transcriptional activation of the CRBP II RXRE in NIH 3T3 cells.

Next, we defined the functional part of the RXRα C terminus in the observed effects by testing chimeras that exchanged both the D (parts 3 and 4) and E (part 5) domains.
When the E domain of RXR\textsubscript{b} was switched with that of RXR\textsubscript{a}, the resultant chimera, RXR\textsubscript{aa}[aab], gave nearly the equivalent amount of transcriptional activity as RXR\textsubscript{aa}[aaa] (compare lane 7 with 1). Therefore, the E domain does not appear to contribute to the observed receptor subtype specificity.

Several lines of evidence derived from our experiments suggest that the D domain (containing chimeric parts 3 and 4) and the A/B domain are critical for the proper functioning of RXR\textsubscript{a} in NIH 3T3 cells. When the D domain of RXR\textsubscript{aa}[aab] is replaced by the D domain of \textbeta, RXR\textsubscript{aa}[bbb], the reporter activity decreases about 75% (compare lane 7 with 6). Furthermore, the D domain of RXR\textsubscript{a} functioned less efficiently when linked to the RXR\textsubscript{b} A/B domain (compare lane 8 with 13). Thus, the maximum effect was only observed when the A/B and the D domains were both from RXR\textsubscript{a} (compare lane 6 with 7 and lane 7 with 12). Taking into account that the E domains are fully exchangeable, these results strongly imply that the D and A/B domains function in concert to achieve optimal transcriptional activation in NIH 3T3 cells.

Based on these results, we further dissected the D domain to identify the portion responsible for this effect. Region D includes the A box and ends at the N terminus of the E domain (Fig. 2, B and C). The A box is a seven-residue region that was originally shown to be critical for binding of the orphan receptor NGFI-B to its response element (14–18, 24). The resolution of the three-dimensional structure of the RXR DBD has shown that the A box is included in a helical region beginning after the second zinc finger of the DBD (25). This helix includes also the so-called T box that forms a dimerization interface critical in the binding of homo- and heterodimeric DBDs to some response elements (14–18, 24, 25).

New chimeras, which exchanged the N terminus, including the A box (part 3), or the C-terminal region (part 4) of the D domain (Fig. 2C), were tested for their transcriptional activation. When part 4 of RXR\textsubscript{b} was used to replace the corresponding portion in RXR\textsubscript{a} (RXR\textsubscript{aa}[aab]), less than 2-fold reduction in overall reporter activity was observed (compare lanes 1 and 4 in Fig. 4A). However, a more dramatic effect was seen when part 3 of RXR\textsubscript{b} was substituted for the analogous region of RXR\textsubscript{a} (RXR\textsubscript{aa}[bb]). Although the transactivation in the presence of hormone was reduced less than 2-fold (Fig. 4A, compare lanes 1 and 5), a 10-fold increase in transactivation in the absence of hormone was detected (Fig. 4B, compare lanes 1 and
Interestingly, this increase in basal activity occurred only when part 3 of the β receptor was substituted for the same part in a receptor containing RXRα A/B domain (RXRα[αββ]) and not when linked to the RXRβ A/B domain (RXRβ[αββ]) (compare lanes 6 and 9 in Fig. 4B). In addition, when the E domain (part 5 of the α receptor was substituted by the E domain of the β receptor (RXRα[ααβ]), an increase of basal activity, although weaker than the chimeras described above, was observed (compare lane 7 with 1, Fig. 4). Nevertheless, the hormone-independent enhancement of basal activity observed in these chimeras demonstrates the functional interaction of the D domain and to some degree the E domain with the A/B domain. Therefore, regions 3 in the D domain and the E domain must be from RXRα to achieve repression of the N-terminal AF-1 in the absence of hormone (Fig. 4A, lane 1).

The importance of the RXRα part 3 for 9cRA-dependent maximal activity in NIH 3T3 cells, in the absence of an increase in basal activity, was also demonstrated using chimeras containing the RXRα A/B domain. When construct 11 (RXRα[αββ]) was tested in NIH 3T3 cells, an 8-fold 9cRA-dependent induction was observed (Fig. 4A, lane 11). However, when part 3 of this construct was replaced with the corresponding part from RXRα (RXRα[ααα]), a further increase of the reporter activity (up to 70-fold 9cRA-dependent induction) was observed (lane 12 compared with lane 11). Although the levels of reporter activity observed in the presence of RXRα[ααα] is much lower than the levels observed with RXRα[αββ], the latter containing the A/B domain of RXRα, it demonstrates again the importance of part 3.

The fact that the D domain of RXRα is involved in both the inhibition of AF1 in the absence of hormone and in hormone-dependent inducibility adds to the growing list of regulatory functions attributed to this region. Other than the T and A boxes, immediately 3’ of the A box is a 70-amino acid region, which has been shown to be responsible for the divergent transcriptional activities between the RARα, -β, and -γ subtypes in response to various retinoids (25).

The data presented here also support the conclusion that there is an AF1 present in RXRα but not in RXRβ (11, 19). Interestingly, Chen and Privalsky (23) have provided evidence that RXRα binds to the CRBP II RXRE as a tetramer, while the A/B domain of RXRβ is involved in inhibiting the tetrameric binding of RXRβ to the CRBP II response element. Differences in DNA binding properties between the receptors have been invoked as the cause for the different transactivation levels observed in SL-2 cells (23).

Our data indicate that, in addition to the intrinsic DNA binding properties of the receptors, interactions with a cell-specific factor(s) or cell type-specific post-translational modifications may be involved, given the differences in response mediated by RXRα and RXRβ observed in P19 and NIH 3T3 cells. Finally, it should be emphasized that, although this study addresses the activities of RXR homodimers, a differential role of RXR subtypes and cell type-specific differences in transcriptional activation might also be found in the regulation of gene expression mediated by RXR heterodimerization with other nuclear receptors.

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