Mechanisms for Synergistic Activation of Thyroid Hormone Receptor and Retinoid X Receptor on Different Response Elements*

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The thyroid hormone receptors (TR) form heterodimers with the retinoid X receptors (RXR) and activate target genes through thyroid-responsive elements (TRE). Heterodimerization elevates the DNA binding efficiency and thus can result in functional synergism between TR and RXR. Here we demonstrate that DNA sequences dictate the cooperative activation between TR and RXR despite the high affinity binding of the heterodimer to those TREs. We provide evidence that the C-terminal activation domain of RXR can modulate the triiodothyronine (T₃) responsiveness of TR/RXR heterodimers on reporter genes without altering the DNA binding properties of the heterodimers. The modulation function of this relatively small region is under the control of specific TRE sequences and promoter context. These data indicate that this C-terminal region of RXR is likely involved in receptor-cellular factor interactions. Finally, we propose that the synergistic activation by TR and RXR is achieved through elevated DNA binding and, dependent on the DNA sequence, the interaction of RXR with other transcription factors.

Members of the nuclear receptor superfamily are ligand-activated transcription factors which interact with their cognate response elements in target genes and thereby regulate diverse aspects of homeostasis, differentiation, and development (1–3). Included in this family of proteins are receptors for steroids, retinoids (RAR and RXR), thyroid hormone (TR), vitamin D₃ (VDR), ecdysone (EcR), and a large number of receptor-like proteins with unknown ligands (orphan receptors). Unlike steroid receptors which usually bind to palindromic response elements as homodimers, TR, RAR, and VDR bind to DNA sequences composed of various arrays of the PuGTT/C/A/A core motif, primarily as heterodimers with RXR (4–8). Characterization of these degenerate response elements led to the conclusion that the orientation and spacing of the core sequence dictates the elective transcription properties for each of these receptors (9–13).

Although TR and RXR can bind to complex response elements (HREs) as monomers and homodimers (14–17), heterodimerization with RXR greatly enhances DNA binding in vitro, and expression of exogenous RXR elevates the hormone responsiveness in transient transfection systems (4–8). Moreover, because of the asymmetric nature of the heterodimers, another level of regulation on DNA binding site selection is introduced (18, 19). Thus, it is very likely that in vivo hormonal induction is a consequence of heterodimerization rather than TR or RAR homodimerization.

However, recent studies have shown that RXR does not always functionally synergize with its heterodimer partners, thus conferring divergent effects on TR, RAR, or VDR-dependent gene regulation (12, 13, 20). Subsequent detailed analysis has suggested that this could result from the following findings: 1) the surrounding sequences and the variation in sequence of the repeated motif in individual HREs have profound influence on their relative activity (18, 21–24); 2) RXR-containing heterodimers induce differential DNA bending when bound to different HREs, which may favor different interactions of heterodimers with other transcription factors thus affecting the activity of these HREs (25); 3) different response elements induce distinct DNA-dependent structural changes within receptors, which may affect the interaction between receptors and other transcription factors (26, 27).

The mechanism for heterodimer synergistic activation is largely unknown. Our previous studies on conformational change of TR (28), together with the above mentioned findings, lead us to hypothesize that the activity of these complex HREs is, at least in part, regulated by a specific interaction of heterodimers with other transcription factors. The synergism between RXR and its heterodimeric partner is mediated by a combination of elevated DNA binding ligand-induced conformational change in heterodimers (28) and specific interactions of heterodimers with other cellular factors. Indeed, recent studies have demonstrated that TR and RAR can interact with TFIIB directly and in addition RAR shows a cooperative transactivating interaction with TFIID which requires an R1A-like activity (29–31).

It has not been demonstrated that the functional cooperativity of RXR with TR or RAR on a certain HRE is modulated by the interaction(s) between heterodimers and other transcription factors. Since it is difficult to obtain direct evidence for such interaction(s), our strategy to substantiate this hypothesis is to identify a specific region in the heterodimeric partners that serves as a target site(s) for the putative interaction(s). If the interaction is required for RXR functional cooperativity, deletion of such a region would abolish the interaction and should impair the synergistic activation of the heterodimer. Our recent studies on mouse RXRβ have delineated a 21-amino acid sequence at the C-terminal end that functions as an activation domain (32). A similar region in RXRα has also been indicated to be involved in receptor activation (33). As opposed to TR or RAR (35), this activation domain can be dissociated

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The abbreviations used are: RAR, retinoid acid receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; VDR, vitamin D₃ receptor; EcR, ecdysone receptor; HRE, hormone response element; MHC, myosin heavy chain; TRE, thyroid-responsive element; T₃, triiodothyronine; CAT, chloramphenicol acetyltransferase.

31436
from the RXRβ E region without abolishing ligand binding and dimerization function of the receptor. Partial proteolytic analysis shows that this region is structurally separated from the rest of the ligand binding domain. This unique arrangement suggests that this region is accessible to other proteins and could be a target site for protein-protein interaction. Thus it may play an important role in RXR and TR synergistic activation.

In this report, we tested the transcription activity of a TR/RXR heterodimer on reporter constructs driven by DR4, TREpal, MHC TRE, and RII enhancer elements in N-tera2 cells. A series of RXR mutants were employed to examine the synergistic activation of TR and RXR on reporters of MHC TRE and RII enhancer. We provide evidence that cooperative DNA binding is sufficient for TR/RXR synergy on the MHC TRE reporter. In addition to the cooperative DNA binding, the RII enhancer reporter required the C-terminal activation domain of RXR for full T3 responsiveness of the heterodimer. The requirement for this specific sequence suggests that the functional synergy between TR and RXR is modulated by receptor interaction with another transcription factor(s). Therefore, RXR not only enhances the DNA binding affinity but also participates in the interaction with other transcription factors, thereby controlling the hormone-dependent activation of a target gene. Finally, we speculate that the totality of synergistic activation by heterodimers is achieved by a combination of enhanced DNA binding and the interaction of the heterodimer partners with other transcription factors.

EXPERIMENTAL PROCEDURES

**Plasmids and Oligonucleotides**—For DNA binding assays, the sense strand of oligonucleotides used were as follows.

- **DR4** gatcc TAG GGGTCA ATATA AGGTCA ATATG a
- **MHC TRE** gatcc TTGGCTCCTG AGGTGAC AAGGAGGACA GC a
- **RII** gatcc AGCGCGTG AGGTCA AATTGAGGTCA AATG a
- **TREpal** gatcc CTAG GGGTCA TGAACCT TGACGA a

Two complementary oligonucleotides for each element were synthesized. All mRXR mutants for in vitro transcription and translation were constructed in pT7Sal (36). pT7βRAR, pT7βRXR, pT7βRXRA2, pT7βRXRA3, and pT7βRXRA4 were described previously (28). pT7βRXRA206-265 was generated by inserting the HindIIICoeRI fragments of corresponding pT7pSal constructs into pT7pRXA. The pBS construct has been described before (6). pG3010 was used for in vitro translation of hTRp (37). The expression vectors for RXR (pRSVXMVXRXR) and RXRA206-265 (pRSVMXRXRA206-265) were created by subcloning HindIII/EcoRI fragments of corresponding pT7Sal constructs into Asp7I8/EcoRI sites of pABGal147 (38). Similarly, pRSVMXRXRA3 and pRSVMXRXRA4 were generated by inserting the HindIII/EcoRI fragments of pT7βRXRA2 and pT7βRXRA3 into SalI/AAsp7I8 sites of pABGal147. pRSVMXRXRA4 was constructed in the same way except that HindIII/XhoI fragment of pT7βRXRA3 was used. When necessary, 5' overhangs were blunt-ended with Klenow enzyme. Expression vectors for rat TRa (pRSVTRa) and RARb (pSV40-RARb) were gifts from Dr. V. Nikodem and Dr. A. De Jean, respectively. DR4 tkCAT and MHC TRE tkCAT/H/N have been described previously (39). Reporters containing TREpal (TREpal tkCAT/H/N) (40) and RII enhancer (pGAM4) (41) have also been described previously. RIIm tkCAT/H/N was not used because of a background problem (41).

**In Vitro Transcription/Translation and Gel Mobility Shift Assays**—Receptor proteins were synthesized using Promega transcription/translation kit. The experimental procedure was described previously (28). Gel mobility shift assays were performed as described previously except that in vitro translated receptors were preincubated with 100 nM T3 and/or 100 nM 9-cis-RA for 15 min at room temperature where specified (28).

**Cell Culture and Transient Transfections**—N-tera2 cells were grown in McCoy's 5A medium supplemented with 10% charcoal-stripped fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. 2 x 10⁶ cells were seeded in each 100-mm dish 24 h before transfection. For TR/RXR transfection assays, 5 μg of reporter, 0.5 μg of TRa, and 1.5 μg of RXRβ expression vector were used per 100-mm dish. Bluescript plasmid was used to bring the total DNA amount to 15 μg. Transfections were carried out as described previously except that 10⁻⁷ m T3 was added 5–6 h after glycerol shock. The CAT activity was determined as described previously (42). For RAR/RXR, transfection assays were done in 12-well plates with 2 x 10⁶ cells/well as described previously (43). In each well, 1 μg of DNA, including 0.3 μg of reporter, 0.2 μg of pCH110 (βgal expression vector from Pharmacia), and 0.5 μg and 10 ng of expression vectors for RAR and RXR, respectively, were added. Cell extracts were prepared with cell lysis buffer (Analytic Luminescence) after 24-h incubation with 5–10 μM RA, and luciferase activity was determined as described previously (44).

**Synergism of TR/RXR**

**A**

| Reporter | TREpal | MHC TRE | RII enhancer |
|----------|--------|---------|--------------|
| **DR4**  | gatcc TAG GGGTCA ATATA AGGTCA ATATG a | N4 |        |
| **MHC TRE** | gatcc TTGGCTCCTG AGGTGAC AAGGAGGACA GC a | N4 |        |
| **RII enhancer** | gatcc AGCGCGTG AGGTCA AATTGAGGTCA AATG a |        |        |
| **TREpal** | gatcc CTAG GGGTCA TGAACCT TGACGA a |        |        |

**B**

![Fig. 1. DNA response elements dictate the cooperative activation of TR and RXR. A schematic representation of various TREs. The sequence of the core motif is indicated by boldface type. Arrows indicate the orientation of the half-sites. B, expression vectors of TR and RXR were co-transfected together with reporter constructs containing DNA, TREpal, MHC TRE, and RII enhancer as indicated into N-tera2 cells. Five to 6 h after glycerol shock, the cells were incubated with or without 100 nM T3 as indicated for 38–42 h. Cell extracts were subsequently prepared and assayed for protein concentration and CAT activity. CAT activities of all samples were normalized to that of βgal (mock expression vector). Results shown are the summary of at least three independent experiments.](image-url)
RESULTS

Interaction of TR with RXR on Various TRE Elements—As summarized in Fig. 1A, various T₃ response elements were used to examine the functional synergism between TR and RXR. DR4 and MHC TRE contain two PuGGA/TCA half-sites arranged in direct repeat orientation and separated by a 4-base pair spacer. TREpal is a synthetic TRE with two palindromic half-sites without spacer and RII enhancer contains only a single half-site (6, 45). As expected, TR was able to heterodimerize with RXR on all these response elements, and the addition of hormones has little effect on heterodimer formation (data not shown). In each of these cases, TR/RXR heterodimers exhibited much higher binding affinity than that of TR homodimer. In order to test if elevated DNA binding affinity of TR/RXR heterodimers also results in increased transcription activity, CAT reporters containing each response element were co-transfected with TR and RXR expression vectors into N-Tera2 cells, a human embryonal carcinoma cell line. As shown in Fig. 1B, functional synergism of TR and RXR was observed on reporters containing MHC TRE and RII enhancer element, in which cases co-transfection of TR and RXR resulted in about a 3- and 5-fold increase of T₃ response, respectively, when compared with that of co-transfecting TR alone. On DR4 and TREpal, however, we could not detect any significant increase.

![Diagram of heterodimerization](image)

**Fig. 2.** Heterodimerization of RXR mutants and TR on the MHC TRE. **A,** a schematic representation of various RXR deletion mutants. Full-length RXR in our constructs consists of 448 amino acids. By comparison with other nuclear receptors, the sequences from amino acids 120–185 and 209–448 indicate the DNA binding and the ligand binding domains of the receptor, respectively (6). The sequences for amino acids 305–418 and 427–448 contain the dimerization and activation function of the receptor, respectively (32). + indicates that the protein is able to heterodimerize with TR in gel mobility shift assay. **B,** similar translation efficiency of different RXR mutants in vitro. 0.5 µl of [35S]methionine-labeled in vitro translated proteins were analyzed on a 10% SDS gel. **C,** unlabeled in vitro translated TR (1 µl) and RXR (2 µl) were incubated for 15 min at room temperature before addition of [32P]labeled MHC TRE in a gel mobility shift assay. NS, nonspecific binding; B, bound; F, free.
of activity by adding RXR, even though varying amounts of expression vector were tested. These results indicate that increased DNA binding affinity alone is not sufficient for synergistic activation of TR/RXR and that the specific target DNA sequence may dictate the functional synergism. Moreover, although TR/RXR heterodimer displayed the weakest DNA binding affinity for the RII enhancer element, this target DNA supported the strongest T3 induction of transcription. This lack of correlation between in vitro DNA binding affinity of TR/RXR heterodimers and their ability to activate target genes suggests that additional factors may influence the T3 responsiveness of TREs.

Heterodimerization of TR and RXR Is Sufficient for TR/RXR Synergism on a Reporter Containing the MHC TRE—To further understand how RXR synergizes with TR, various RXR deletion mutants were constructed. As summarized in Fig. 2A, RXRa206–265 contains an internal deletion which destroyed the ligand binding and transactivation function of the receptor; RXRa2, RXRa3, and RXRa4 are C-terminal truncation mutants in which the C-terminal activation domain of RXR is removed (33). It has been shown that RXRa2 is capable of forming both homodimers and heterodimers efficiently, RXRa3 can form heterodimers but not homodimers, and RXRa4 cannot form either homodimers or heterodimers. These mutants and wild-type RXR were synthesized in parallel using rabbit reticuloocyte lysates and [35S]methionine. SDS-gel electrophoresis indicated that similar levels of RXR mutants were produced (Fig. 2B). In vitro translated unlabelled receptors were then used in gel mobility shift assays to examine the heterodimer formation of TR with these mutants on the MHC TRE. As shown in Fig. 2C, all these mutants, except for RXRa4, which has its heterodimerization domain deleted, could heterodimerize with TR and bind to the DNA response element with increased binding affinity. Similar results were also obtained when these mutants were tested with RAR (data not shown).

To test for the functional synergism, expression vectors of these RXR mutants and TR were co-transfected into N-Tera2 cells together with a CAT reporter containing MHC TRE. As indicated in Fig. 3, co-transfection of TR with RXRa206–265, RXRa2, or RXRa3 all resulted in an enhanced T3 response when compared with that of TR alone. The observed synergistic effects of these mutants with TR were similar to that of wild-type RXR. Co-transfection of TR with various amounts of RXRa4, however, did not reveal transcriptional synergism. Thus, mutants that could form heterodimers were able to functionally synergize with TR, whereas the RXRa4 mutant that could not form a heterodimer did not demonstrate synergism. These data reveal a good correlation of heterodimer formation and functional synergism of TR and RXR mutants on the MHC TRE. Thus cooperative DNA binding of TR and RXR may be sufficient for synergistic transactivation on the MHC TRE containing reporter.

C-terminal Activation Domain of RXR Is Required for Functional Synergism of TR and RXR on a CAT Reporter Containing the RII Enhancer—Similar to that on the MHC TRE, these RXR mutants were also able to heterodimerize with TR on the RII enhancer element, albeit weakly (6). Except for RXRa4, all these RXR mutants could cooperate with TR in DNA binding. To examine functional synergism of TR and RXR on the RII enhancer element, a CAT reporter pLdl.4k was used. TR and RAR have been shown to activate transcription of this reporter through the RII enhancer in a T3- and RA-dependent manner, respectively, after co-transfection into N-Tera2 cells (6, 41). Transient transfection assays were carried out with TR and RXR mutants. In contrast to what we observed with the MHC TRE, co-transfection of RXRac2 or RXRac3 with TR conferred only an additive effect on T3 responsiveness (Fig. 4A). In contrast, co-transfection of RXRa206–265 with TR presented a strong synergistic activation comparable with that of wild-type RXR (Fig. 4A). The lack of synergism of RXRac2 and RXRac3 was not due to insufficient expression of these proteins (data not shown). Moreover, since RXRa3 is not able to form a significant amount of homodimer, it is unlikely that the lack of synergism between the TR and RXR mutants was due to direct competition for the DNA binding site of the mutant homodimers. Similar to what we obtained with the MHC TRE, RXRa4 did not synergize with TR on pLdl.4k. These data suggest that, with pLdl.4k as a target, synergistic activation between TR and RXR requires not only the cooperative DNA
binding of TR and RXR but also the intact C-terminal activation domain of RXR.

To examine if the synergistic requirement for the C-terminal activation domain of RXR is TR-specific, RAR and RXR mutants were co-transfected into N-Tera2 cells together with pLd1.4k-Luc. As shown in Fig. 4B, although co-transfection of wild-type RXR increased RA-dependent induction of RAR, co-transfection of RXRΔc2 or RXRΔc3 failed to produce any enhancement. These data confirm that for full synergistic activation of RXR with other receptors through the RII enhancer on pLd1.4k, the C-terminal activation domain of RXR is required in addition to the regions that conduct cooperative DNA binding. The requirement of a specific region of RXR for the complete activation of heterodimers indicates that interaction of heterodimers with another cellular factor(s) may be critical.

**DISCUSSION**

RXR plays a central role in mediating many hormonal signaling pathways through heterodimer formation with TR, RAR, VDR, and the orphan receptors, COUP-TF and PPAR subgroup (4-8, 32, 39, 46-48). It has been shown previously that heterodimerization elevates the binding of these receptors to a number of natural and synthetic DNA elements containing PuGGT(C/A)A or a related motif. Therefore it is believed that by modulating receptor DNA binding, RXR potentiates transcriptional activation by these receptors.

Many different response elements for TR and RAR have been identified in promoter regions of various target genes, each with different half-site or spacer sequences and locations with respect to the transcription start sites. Despite what appears to be similar effects of RXR in promoting heterodimer DNA binding (17, 18), the potency of RXR in synergistic activation with these receptors differs greatly (12, 13, 20). Many observations have been suggested to explain this complicated situation (21-23, 25-27, 34), all of which indicates that the functional synergism may be achieved through not only cooperative DNA binding but also via proper interactions of heterodimers with
other transcription factors.

As shown in Fig. 4, deletion of a small region from the RXR C terminus (RXRAc2 and RXRAc3) diminished its synergistic transactivation function on pLdl.1k, but did not impair its heterodimerization ability with TR or RAR. Interestingly, we have shown that this same C-terminal region contains a functional activation domain of RXR. In this case, cooperative DNA binding is not sufficient to confer full transactivation function to the heterodimer. Deletion of another internal region (RXRA206–265), however, did not lessen the functional synergy between TR and RXR. All these internal or C-terminal deletions abolished the transactivation potential of RXR (33), but did not affect the heterodimerization function of the receptor (6). One major difference between RXRA206–265 and RXRAc2 or RXRAc3 is that RXRA206–265 contains the C-terminal activation domain of the receptor. Thus, the most likely explanation for the functional discrepancy of these mutants is that a specific interaction with other transcription factor(s), of which the C-terminal region of RXR is the target site, is required for synergistic activation. We wish to point out that these results do not exclude the possibility that there is another potential target site(s) within RXR for protein-protein interaction in heterodimer formation. In addition, the complexity of the pLdl.1k promoter may also modify the outcome of the heterodimer transactivation by facilitating the interaction of RXR C-terminal activation domain with other transcription factors.

It is intriguing that the mechanism for TR/RXR synergism on MHC TRE reporter is different. In contrast to what we observed with pLdl.1k, deletion mutants, RXRAc2, RXRAc3, and RXRA206–265, all potentiate TFARXR synergism on pLdl.4k, but did not impair its heterodimerization ability with TR or RAR. Interestingly, we have shown that this same C-terminal region contains a functional cooperativity. However, it does not rule out the interaction of RXR with other transcription factors. It is possible that on the MHC TRE, enhanced synergism between TR and RXR implies another dimension of transcriptional regulation. Since TR and RXR may interact with different cellular factors, heterodimerization of TR with RXR not only offers cross-talk between different hormonal signaling pathways, but also ties these different cellular factors together. Thus, in addition to those factors which interact with TR, this induction on a specific TRE also may be regulated by factors that only interact with RXR. It may therefore impose another level of regulation to fine-tune target gene expression.

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Synergism of TR/RXR

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