Novel Roles of Retinoid X Receptor (RXR) and RXR Ligand in Dynamically Modulating the Activity of the Thyroid Hormone Receptor/RXR Heterodimer*

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Many members of the type II nuclear receptor subfamily function as heterodimers with the retinoid X receptor (RXR). A permissive heterodimer (e.g. peroxisome proliferator-activated receptor/RXR) allows for ligand binding by both partners of the receptor complex. In contrast, RXR has been thought to be incapable of ligand binding in a nonpermissive heterodimer, such as that of thyroid hormone receptor (TR)/RXR, where it has been referred to as a silent partner. However, we recently presented functional evidence suggesting that RXR in the TR/RXR heterodimer can bind its natural ligand 9-cis-RA in cells. Here we extended our study of the interrelationship of TR and RXR. We examined the potential modulatory effect of RXR and its ligand on the activity of TR, primarily using a Gal4-TR chimera. This study led to several novel and unexpected findings: 1) heterodimerization of apo-RXRα (in the absence of 9-cis-RA) with Gal4-TR inhibits T3-mediated transactivation; 2) the inhibition of Gal4-TR activity by RXRα is further enhanced by 9-cis-RA; 3) two different RXR subtypes (α and β) differentially modulate the activity of Gal4-TR; 4) the N-terminal A/B domains of RXR α and β are largely responsible for their differential modulation of TR activity; and 5) the RXR ligand 9-cis-RA appears to differentially affect T3-mediated transactivation from the Gal4-TR/RXRα (which is inhibited by 9-cis-RA) and TRE-bound TR/RXRα (which is further activated by 9-cis-RA) heterodimers. Taken together, these results further support our recent proposal that the RXR component in a TR/RXR heterodimer is not silent and, more importantly, reveal novel aspects of regulation of the activity of the TR/RXR heterodimer by RXR and RXR ligand.

The thyroid hormone receptors (TRs)* and the retinoid X receptors (RXRs) are members of the nuclear receptor superfamily, which consists of a large number of special transcription factors whose activities in many cases are regulated by their cognate ligands (1). The superfamily is generally divided into two groups. Type I receptors are classic steroid receptors that mediate the actions of steroid hormones such as glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens. TRs and RXRs belong to the type II group, whose additional members include the retinoic acid receptors (RARs), the receptor for 1,25-(OH)2 vitamin D3 (VDR), the peroxisome proliferator-activated receptors (PPARs), and many orphan receptors whose ligands (if any) remain to be defined. Members of the superfamily exhibit similar domain structures. Typically, a receptor molecule consists of a highly variable N-terminal A/B domain, a highly conserved central DNA binding domain (DBD; region C) that is characterized by two signature zinc fingers, and a C-terminal ligand binding domain (LBD; regions D, E, and F) that is less conserved at the amino acid sequence level but exhibits remarkably similar overall structure (1–4).

Several distinct features exist in the actions of type I and type II receptors. For example, type I receptors are usually associated with heat shock protein chaperones in the absence of ligand and do not bind DNA (2, 5). The binding of a ligand dissociates the chaperones and allows for type I receptors to engage in DNA binding and transcriptional regulation. In contrast, type II receptors are not known to be chaperone-associated and thus can constitutively bind to DNA with or without ligands (6–8). A functional consequence of constitutive DNA binding by certain type II receptors is that in addition to their ligand-mediated transactivation function, they can also mediate transcriptional repression in the absence of ligand (9–12). Another difference among type I and type II receptors is that type I receptors primarily act as homodimers that bind to their palindromic hormone response elements (HREs) (2, 5), whereas type II receptors can bind to DNA as monomers, homodimers, and heterodimers (7, 13). The corresponding HREs are also complex for type II receptors, which can be organized as direct repeats (DR), inverted repeats and everted repeats (4). A particularly intriguing feature of type II receptors is that many of them function as heterodimers with RXR (4). In this regard, RXR appears to have a central role in the actions of those type II receptors, such as TRs, RARs, VDR, and PPARs, all of which are believed to function in the context of heterodimers with RXR (4). The primary dimerization interface lies in the ligand binding domain (LBD) of the receptor partners, whereas a region from the DBD is also involved (4). Interestingly, a number of recently identified orphan receptors (e.g. LXR, FXR, PXR, and BXR) were also found to heterodimerize with RXR, extending the partners for RXR heterodimerization (14). It is unclear why so many type II receptors engage RXR as their functional partner, although it has been proposed that this may reflect an ancient requirement for heterodimerization during the evolution of nuclear receptors.

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The abbreviations used are: TR, thyroid hormone receptor; TRE, thyroid hormone response element; RXR, retinoid X receptor; RAR, retinoic acid receptor; VDR, 1,25-(OH)2 vitamin D3 receptor; PPAR, peroxisome proliferator-activated receptor; DBD, DNA binding domain; DR, direct repeats; LBD, ligand binding domain; 9-cis-RA, 9-cis-retinoic acid; hRXR, human RXR; G4/80V, Gal4-TR(480)-VP16; CAT, chloramphenicol acetyltransferase.
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(15). In this regard, it is interesting to note that whereas type II receptors that form partners with RXR can bind their ligands autonomously, their binding to specific HRE(s) usually requires or is facilitated by heterodimerization (4, 14).

Aside from its role in facilitating DNA binding, heterodimerization between a type II receptor and RXR provides the potential for the resulting heterodimer to function as a new entity, to respond to the binding of both two different ligands. Indeed, this is the case in permissive heterodimers such as PPAR/RXR, which is activated by both PPAR and RXR ligands (16). In contrast, RXR heterodimers with TR, RAR, and VDR and VDR have been thought to be nonpermissive, since the treatment of the RXR ligand alone usually fails to elicit transactivation from these heterodimers (4, 17, 18). This observation had led to a silent partner model for RXR in these nonpermissive heterodimers, where it is proposed that the allosteric control by TR or RAR somehow prevents RXR from engaging in ligand binding. However, a number of studies over the last several years have provided evidence indicating that RXR in a RAR/RXR heterodimer can bind its ligand and mediate its functional or biological consequences (19–24). In a recent report, we presented a functional evidence indicating that in the TR/RXR heterodimer, RXR can bind its natural ligand 9-cis-RA in cells and thus is not a silent partner (25). Interestingly, although ligand binding by RXR does not directly elicit transactivation from the apo-TR/ligated RXR heterodimer, it promotes the dissociation of a corepressor(s) from TR (25). This may in turn dynamically modulate the transcriptional output of the heterodimer by attenuating the repression function of apo-TR and/or enhancing the activation function of liganded TR.

The notion that RXR is not a silent partner raises the possibility that RXR and/or its ligand may play a role in the TR/RXR heterodimer. TR clearly plays a major role in defining the transcriptional property of the TR/RXR heterodimer by directly mediating repression (in the absence of T3) and activation (in the presence of T3) (12, 18, 25, 26). In contrast, the role of RXR (other than facilitating DNA binding) and its ligand is not well characterized. This is in part due to the fact that the silent partner model for RXR in the TR/RXR heterodimer has been a rather prevalent notion in the field. Only recently have questions been raised about this model by any systematic study (25). An additional complexity is that by using a traditional TRE-based reporter assay, it is difficult to differentiate the relative contributions of several potential mechanisms by which RXR could affect reporter activity (which include, for example, facilitation of DNA binding by TR, competition with TR for the DNA binding site, and the potential direct contribution to transcriptional regulation). Thus, in this report, we examined the potential modulatory effect of RXR on the activity of TR, primarily using a Gal4-TR chimera model system. The results from these studies provide further support for our recent proposal that RXR is not a silent partner in the TR/RXR heterodimer and, more importantly, reveal novel roles of RXR and RXR ligand in regulating the activity of the TR/RXR heterodimer.

EXPERIMENTAL PROCEDURES

Plasmid Constructs for Transfection Studies—The Gal4-responsive reporters pMC110 and G5-tk-CAT were described previously (9, 12). The plasmid Gal4-TR has been described previously, and expresses the ligand-binding domain of cTRs (residues 120–408) fused in frame to the DBD of Gal4 (9). The Gal4-ER plasmid expressing a Gal4 fusion of the mERs LBD was obtained from Malcolm Parkar (27). Plasmids expressing hRXRα and hRXRβ in mammalian cells (pCMV-hRXRα and pCMV-hRXRβ) were obtained from Ron Evans. The empty expression vector (referred to as pCMX) that contains no cDNA insert was used as the control. Plasmids expressing full-length cTRs, the LBD of cTRs (residues 120–408), the LBD of mRXRα (residues 185–438), and the control empty expression vector pEX0, as well as plasmids expressing Gal4-TR-VP16 (GTV) and Gal4-TR(408)-VP16 (GT(408)V), have been described previously (7, 9, 28). The plasmid expressing C-SMRT, a dominant negative form of SMRT, was provided by Ron Evans (10). The TRE-DR4A-CAT reporter was described previously (29). It contains a chloramphenicol acetyltransferase (CAT) gene under the control of the ΔMTV basal promoter linked to a synthetic direct repeat (separated by a 4-bp gap, or DR4) TRE sequence (AGGACACagggAGGAC, where the core repeated sequence is shown in capital letters). The vector expressing cPSF, a dominant negative form of the DBD-interacting corepressor PSF, was described previously (30).

Vectors expressing RXRα1A/B and RXRβ3A/B were generated by PCR-based cloning. The PCR primers were AU1 (5′-GCC GAC GTG AAG AGG CTT TAT ACC ATG GCT TGC GCC ATC GGC GAC CGC CGT GGG GAC ACC GTC TCT CGT GGC ACC CCA GGA AGA AGA-3′), BU1 (5′-GCC GAC GTG AAG AGG CTT TAT ACC ATG GCT TGT GCA ATC GGC GAC AGA AGA-3′), and BD1 (5′-CCG GAC GTG GCT ACC TCT GAG CTC CCA GGT CAT GAT-3′). The PCR templates were pCMV-hRXRα and pCMV-hRXRβ, respectively. Primer pairs AU1 + AD1, and BU1 + BD1, were used to PCR-amplify fragments encoding the entire DBD-LBD regions of hRXRα and hRXRβ, respectively, with their corresponding A/B regions removed. In each case, the resulting PCR products were digested with HindIII and Acc65I, gel-purified, and ligated to a pEX-based vector digested with the same pair of enzymes. The resulting pEX-RXRα1A/B and pEX-RXRβ3A/B vectors were confirmed by sequence analysis. Each vector contained a desired TRE expression unit linked to an optimal core repeated sequence originally present in pEX (28). The vector expressing the A/B domain of hRXRα was also generated by PCR-based cloning. Primers XRA1 (5′-GCC GAC GTG CTC GAG ATC CCA TGC GCC ATC TGC GGG GAC CGC CGT GGG TAT ACC TGG TG-3′) and XRA2 (5′-GCC GAC GTG GCT ACC TCT GAG CTC CCA GGA AGA-3′) were used to PCR-amplify the region encoding the entire A/B domain of hRXRα (residues 1–154), using pCMV-hRXRα as the template. The PCR product was digested with Xhol and Acc65I, gel-purified, and ligated to a green fluorescent protein vector digested with the same pair of enzymes. The resulting green fluorescent protein-hRXRα/B vector was confirmed by sequence analysis. The empty green fluorescent protein vector was used in control transfections when applicable.

Cell Culture and Transfections—HeLa cells were cultured as previously described (9). Plasmids used for transfection studies are described above. Unless specified otherwise in figure legends, cells were transfected by calcium phosphate coprecipitation as described in earlier studies (31, 32). In some cases, cells were transfected using Geneporter 2 (Gene Therapy Systems) as described earlier (32). The identity and purity of the reporter constructs were confirmed by restriction analysis. The empty green fluorescent protein vector was used in control transfections when applicable.

RESULTS

Apo-RXRαs Negatively Modulates Gal4-TR-mediated Transactivation—RXR is generally believed to play a subordinate role in the TR/RXR heterodimer, where it facilitates the binding of TR to certain TREs such as the DR4 sequence (4). Indeed, the transcriptional property of the TR/RXR heterodimer is mainly dictated by TR, since the presence or absence of T3 determines whether the heterodimer functions as an activator or a repressor (12, 18, 25, 26). In contrast, whether RXR makes any direct contribution to the transcriptional output of the
We utilized a Gal4-TR chimera and a Gal4 reporter model system to study the potential effect of RXR. In a classic TRE-based reporter assay system, the effect of RXR on TR-mediated reporter activation may occur through several mechanisms, including facilitation of DNA binding by TR, competition with TR for the DNA binding site, and the potential direct contribution to transcriptional regulation. In contrast, the use of a TR for the DNA binding site, and the potential direct contribution to the transcriptional output of the TR/RXR heterodimer would be expected to result in diminished or no transactivation in the absence of exogenously expressed RXR.

We recently reported functional evidence suggesting that RXR in the TR/RXR heterodimer can bind its ligand 9-cis-RA in cells and thus is not a silent partner (25). In light of this, we further examined the effect of RXR on TR (Fig. 1). The observed 2–3-fold inhibition of Gal4-TR activity by apo-RXR was consistently observed in different experiments. This result suggests that apo-RXR elicits a negative modulatory effect on the activity of liganded TR, at least under the described experimental conditions.

9-cis-RA Further Enhances RXRα-mediated Inhibition of Gal4-TR Activity—We recently reported functional evidence suggesting that RXR in the TR/RXR heterodimer can bind its natural ligand 9-cis-RA in cells and thus is not a silent partner (25). In light of this, we further examined the effect of RXRα on the activity of Gal4-TR in the presence of 9-cis-RA. HeLa cells were transfected with pMC110 and Gal4-TR, along with either pCMX-RXRα or a control vector (pCMX), to examine T3-mediated transactivation. In the absence of exogenously expressed RXRα, Gal4-TR strongly activated the reporter in the presence of T3, and this activation was not affected by 9-cis-RA (Fig. 2A). Coexpression of RXRα led to several observations. First, incubation with 9-cis-RA alone did not activate the reporter (Fig. 2A). This is consistent with previous results from us and others suggesting that a TR/RXR heterodimer cannot be directly activated by the RXR ligand alone (18, 25). Second, RXRα was found to inhibit T3-mediated transactivation by Gal4-TR in the absence of 9-cis-RA (Fig. 2A), confirming our earlier observations (e.g. in Fig. 1). Third, to our surprise, incubation with 9-cis-RA led to further enhancement RXRα-mediated inhibition of Gal4-TR activity (Fig. 2A). Thus, whereas apo-RXRα reduced the activity of Gal4-TR by about 2-fold, the treatment of 9-cis-RA led to a further reduction of about 3–4-fold. Overall, liganded RXRα down-regulated the activity of Gal4-TR by about 8-fold (Fig. 2A).

One potential explanation for the inhibition of Gal4-TR-mediated transactivation by liganded RXRα is squelching (33), where liganded RXRα (when overexpressed) may compete with TR for limited endogenous coactivators. To test this, a titration experiment was carried out with different doses of transfected RXRα ranging from 100 ng to 1 µg. As shown in Fig. 2B, a potent inhibition (about 5-fold) of Gal4-TR activity was observed even when only 100 ng of RXRα was cotransfected, suggesting that the inhibition is unlikely to be due to squelching. Furthermore, in a parallel titration experiment where TR instead of RXRα was cotransfected, no inhibition on Gal4-TR activity was detected even at 1 µg of TR (Fig. 2B). Taken together, the results of our titration experiments suggest that the inhibition of Gal4-TR-mediated transactivation by liganded RXRα is not due to squelching per se.

Heterodimerization Mediates the Inhibition of Gal4-TR Activity by Liganded RXRα—In light of our recent finding that RXR in the TR/RXR heterodimer can bind its ligand 9-cis-RA in cells (25), one possible explanation for our results in Fig. 2 is that heterodimerization mediates the inhibition of Gal4-TR activity by liganded RXRα. As a test of this hypothesis, we examined whether the activity of Gal4-ER (in the presence of E2) is affected by RXRα. Since ER does not form heterodimers with RXRs, the hypothesis predicts that RXRα should not inhibit the activity of Gal4-ER. This is indeed the case (Fig. 3A). Under similar transfection conditions wherein liganded RXRα significantly inhibited Gal4-TR activity (about 4-fold), no inhibition of Gal4-ER activity was observed (Fig. 3A). Apo-RXRα (in the absence of 9-cis-RA) also had no effect on Gal4-ER activity (data not shown). Thus, the selective inhibition of the activity of Gal4-TR (but not Gal4-ER) by RXRα further argues against the “squelching” model.

If the inhibition of Gal4-TR activity by liganded RXRα involves heterodimerization between TR and RXR, overexpression of the TR LBD in trans should compete with Gal4-TR for binding to RXRα and thus may relieve the inhibition. To test this, we transfected HeLa cells with Gal4-TR, the reporter pMC110, and pCMX-RXRα (or the control pCMX) to examine the inhibition of Gal4-TR-mediated transactivation by liganded RXRα while an excess amount (about 3 µg) of pEX-TR LBD or the control vector pEX0 was also cotransfected. As shown in Fig. 3B, in the control experiment cotransfected with pEX0, the activity of Gal4-TR was significantly inhibited (about 4-fold) by liganded RXRα. However, cotransfection of the TR LBD completely abolished the inhibition (Fig. 3B). Taken together, our results in Fig. 3 suggest that inhibition of Gal4-TR activity by liganded RXRα is mediated by heterodimerization between the two receptors.

Liganded RXRα Does Not Activate the GTV Chimera—We have previously shown that a liganded RXR LBD can heterodimerize with a Gal4-TR-VP16 (GTV) chimera and induce a (at least transient or dynamic) dissociation of corepressor(s) from the TR moiety, resulting in derepression (25). The GTV chimera is ordinarily inactive due to a cellular corepressor(s) associating with the TR moiety (9, 25). However, a derepression effect by liganded RXR LBD enables transactivation by GTV (25). Given this, it is somewhat surprising to find that liganded full-length RXRα in heterodimerizing with Gal4-TR, inhibits T3-mediated transactivation. However, one major difference in
these experiments is that derepression of GTV was observed
with a liganded RXR LBD (25), whereas inhibition of Gal4-TR
activity was detected for a liganded full-length RXR
(Figs. 2 and 3). Thus, one possibility is that heterodimerization
with Gal4-TR reveals a cryptic repression function in the liganded
full-length RXRα, which resides outside its LBD region. Con-
sistent with this interpretation, we found that the GTV chi-
mera was not activated by heterodimerization with a liganded
full-length RXRα, despite the fact that it was activated by a

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**Fig. 2.** A, the RXR ligand 9-cis-RA further enhances RXRα-mediated inhibition of Gal4-TR activity. HeLa cells were transfected with 1 μg of the pMC110 reporter and 50 ng of the Gal4-TR expression vector. The vector expressing RXRα or the control vector pCMX (350 ng each) was cotransfected as indicated. CAT activities were determined for cells without ligand (open columns), with 9-cis-RA only (hatched columns), with T3 only (filled columns), and with T3 + 9-cis-RA (wavy columns). B, liganded RXRα but not liganded TR mediates inhibition of Gal4-TR activity. HeLa cells were transfected with 1 μg of the pMC110 reporter and 50 ng of the Gal4-TR expression vector to examine the activity of Gal4-TR in the presence of T3. At the left, an increasing amount of RXRα expression vector (from 100 to 1000 ng) or the control vector pCMX (at an equivalent amount) was included in the transfection (in the presence of 9-cis-RA) to examine liganded RXRα-mediated inhibition of Gal4-TR activity. At each data point, the -fold inhibition was calculated as in Fig. 2B. At the right, overexpression of the TR LBD in trans abolishes liganded RXRα-mediated inhibition of Gal4-TR activity. HeLa cells were transfected with 1 μg of the pMC110 reporter and 50 ng of the Gal4-TR expression vector, together with either the RXRα expression vector or the control vector pCMX (250 ng each) to examine liganded RXRα-mediated inhibition of Gal4-TR activity. When indicated, a vector expressing the TR LBD or the control vector pEX0 (3 μg each) was cotransfected. The -fold inhibition was calculated as in Fig. 2B.
liganded RXR LBD (Fig. 4). In addition, we also found that the RXR LBD did not inhibit Gal4-TR-mediated activation in the absence or presence of 9-cis-RA (data not shown), consistent with the notion that the cryptic repression function resides outside the LBD.

Constitutive Activity of GT(408)V Is Not Inhibited by Liganded RXRα—As discussed above, our findings that liganded RXRα inhibits the activity of Gal4-TR and fails to derepress the GTV chimera can be best explained in a model wherein heterodimerization with Gal4-TR reveals a cryptic repression function in the liganded full-length RXRs that resides outside its LBD region. However, an alternative possibility is that heterodimerization between liganded RXRα and TR may somehow interfere with the binding of the Gal4 DBD moiety to its response element, thus reducing the activation of the Gal4 reporter.

To rule out this possibility, we examined the potential effect of liganded RXRα on the constitutive activity of the GT(408)V chimera. The GT(408)V chimera is similar to the GTV chimera used in our earlier studies and in Fig. 4, except that its TR moiety is transcriptionally inactive due to a cellular corepressor(s) associating with the TR moiety that inhibits or masks the activation function of VP16 (9, 25). Consistent with our earlier results, GTV is activated by the liganded RXR αLBD (25). However, liganded full-length RXRα fails to activate the GTV chimera.

activity of Gal4-TR (which is due to the activation function of liganded TR), but not the activity of GT(408)V (which is due to the activation function of VP16), is inhibited by liganded RXRα, as shown in Fig. 5. Consistent with our earlier results, apo-RXRα negatively modulates the activity of Gal4-TR, as shown in Fig. 6. In the control transfection, 9-cis-RA had little effect on T3-mediated transactivation by Gal4-TR. Consistent with our earlier results, apo-RXRα signifi-

![Fig. 4. Liganded full-length RXRα does not activate the GTV chimera.](image)

![Fig. 5. Constitutive activity of the GT(408)V chimera is not inhibited by liganded RXRα.](image)
RXR and Its Ligand in the TR/RXR Heterodimer

**Fig. 6. RXRα and β Differentially Modulate Gal4-TR Activity.** HeLa cells were transfected with pMC110 (800 ng) and the Gal4-TR expression plasmid (60 ng), together with either a vector expressing RXRα or RXRβ or the control vector pCMX (300 ng each) to compare the effects of RXRα and RXRβ on Gal4-TR activity. CAT activities were determined for cells without ligand (open columns), with 9-cis-RA only (hatched columns), with T3 only (filled columns), and with T3 + 9-cis-RA (wavy columns). See “Results” for details.

Significantly inhibited the activity of Gal4-TR (about 3-fold), whereas a more potent inhibition (about 10-fold) was observed for liganded RXRα (Fig. 6). Interestingly, cotransfection of RXRβ had little effect on Gal4-TR activity in the absence of 9-cis-RA and only led to a very minor reduction in Gal4-TR activity in the presence of 9-cis-RA (Fig. 6). Under similar transfection conditions with the same amount of pCMX-RXRβ, we observed a robust 9-cis-RA-mediated activation of an RXR-responsive reporter (data not shown), confirming that RXRβ is expressed in our transfected cells. Thus, the two subtypes of RXRs examined (α and β) manifest markedly distinct properties in modulating the activity of their heterodimeric partner Gal4-TR. In the absence of 9-cis-RA, apo-RXRα inhibits the activity of Gal4-TR, whereas apo-RXRβ shows little effect. Liganded RXRα potently down-regulates Gal4-TR activity (by about 10-fold; see Fig. 6), but liganded RXRβ only shows a very minor effect (less than 1.5-fold).

9-cis-RA Differentially Modulates the Activities of Gal4-TR/RXRα and TRE-bound TR/RXRα—Our study with Gal4-TR revealed an unexpected role for RXRα in regulating the transcriptional output of the TR/RXR heterodimer. Thus, in a Gal4-TR/RXRα heterodimer, apo-RXRα appears to negatively modulate TR-mediated transactivation (Fig. 1). Moreover, transactivation by the Gal4-TR/RXRα heterodimer (in the presence of T3) was found to be significantly inhibited by the RXR ligand 9-cis-RA (Figs. 2 and 6). Since Gal4-TR/RXRα represents a heterodimer not bound to TRE, we were interested in testing whether the modulation of TR activity by RXRα observed in this model system also extends to a TRE-bound heterodimer.

To test whether apo-RXRα negatively modulates TR activity in the context of a TRE-bound heterodimer, we transfected HeLa cells with the TRE-DR4A-CAT reporter, together with either 100 ng of TR alone or 100 ng of TR and 100 ng of RXRα. A relatively small amount of RXRα was used in the transfection to reduce the chance of potential competition of DNA binding by the expressed RXRα. As shown in Fig. 7A, cotransfection of RXRα led to a reduction in T3-mediated activation of the reporter. This result is consistent with the negative modulatory effect of apo-RXRα on TR activity indicated by our earlier study using Gal4-TR.

In contrast, liganded RXRα appears to manifest very different effects on TR activity in the contexts of Gal4-TR/RXRα and TRE-bound TR/RXRα heterodimers. Earlier studies in this report (e.g. in Figs. 2 and 6) showed that T3-mediated activation of the Gal4-TR/RXRα heterodimer was significantly inhibited by the RXR ligand 9-cis-RA (usually by about 3-fold). In contrast, we have previously reported that in a TRE-bound TR/RXRα heterodimer, 9-cis-RA further stimulated T3-mediated activation (by about 3-fold) (25). An example of a comparison study highlighting the differential effects of liganded RXRα in these two different heterodimers is shown in Fig. 7B. Thus, the transcriptional activities of a heterodimer bound to TRE (TRE-bound TR/RXRα) and that not bound to TRE (Gal4-TR/RXRα) are differentially modulated by 9-cis-RA, suggesting a novel role for DNA binding in determining the effect of an RXR ligand on the activity of the TR/RXR heterodimer.

We have previously shown that in the context of a TRE-bound TR/RXRα heterodimer, liganded RXRα facilitates the dissociation of co-repressor(s) from TR, which may in turn attenuate unliganded TR-mediated repression and/or enhance liganded TR-mediated activation (25). The fact that 9-cis-RA further stimulates T3-mediated transactivation by a TRE-bound TR/RXRα heterodimer is consistent with a derepression effect by liganded RXR that we previously reported. However, in light of the new finding that apo-RXRα negatively modulates TR activity (Fig. 7A), the stimulation of T3-mediated activation from a TRE-bound TR/RXRα heterodimer by 9-cis-RA could result from several not mutually exclusive mechanisms: 1) facilitation of co-repressor dissociation from TR, 2) reversal of the inhibition previously imposed by apo-RXRα, and 3), direct participation of the liganded RXR in coactivator recruitment. The mechanism(s) notwithstanding, this stimulatory effect by 9-cis-RA is only observed for the TRE-bound heterodimer (Fig. 7B), suggesting that conformational change induced by TR binding plays an important role in the process. Indeed, in the context of a heterodimer not bound to TRE, liganded full-length RXRα fails to derepress GTV (Fig. 4), whereas 9-cis-RA enhances RXRα-mediated inhibition of Gal4-TR activity (Figs. 2A and 6).

The Effect of 9-cis-RA on a TRE-bound TR/RXRα Heterodimer—In contrast with RXRα, RXRβ was found to have little effect on Gal4-TR activity in the absence or presence of 9-cis-RA (see Fig. 6). The potential effect of RXRβ was also tested on a TRE-controlled reporter in experiments similar to that shown in Fig. 7. As shown in Fig. 8, consistent with the minimal effect of RXRβ observed with the Gal4-TR system, we found that cotransfection of RXRβ did not significantly affect T3-mediated activation from the TRE-controlled reporter in the absence or presence of 9-cis-RA (Fig. 8).

Interestingly, in cells cotransfected with either the pCMX control or RXRβ, we observed a minor but nevertheless consistent stimulation of reporter activity in the presence of both T3 and 9-cis-RA compared with just T3 alone (by about 30%; see Fig. 8). This increase in reporter activity in control cells is not due to cross-activation of the TRE-controlled reporter by endogenous retinoid receptors, since treatment with 9-cis-RA alone (in the absence of T3) resulted in no activation (Fig. 8). Since HeLa cells contain endogenous RXR(s) (39), the further stimulation of T3-mediated reporter activation by 9-cis-RA in the absence of any cotransfected RXR is probably due to the
binding of the TRE by heterodimers formed between the transfected TR and endogenous RXR(s). This result is nevertheless consistent with the notion that a TRE-bound TR/RXR heterodimer can be superactivated by the presence of both T3 and 9-cis-RA, as suggested by our earlier and current studies (25) (Fig. 7B). In this regard, it is possible that the apparent lack of effect by cotransfected RXRα/H9252 in the presence of 9-cis-RA (Fig. 8) is due to the fact that the resulting TR/RXRα/H9252 heterodimer, when bound to the TRE, manifests a similar overall property to the heterodimer(s) formed between TR and endogenous RXR(s). Indeed, using a biochemical purification approach, Leid et al. (40) identified hRXRα/H9252 as the nuclear protein in HeLa cells that facilitates the DNA binding of TRs and RARs, suggesting that cellular hRXRα/H9252 proteins are readily available for heterodimerization.

Role of the N-terminal A/B Domain in Differential Regulation of TR Activity by RXRα/H9251 and RXRα/H9252—Our study in Fig. 6 shows that the two subtypes of RXRs (α and β) manifest markedly distinct properties in modulating the activity of Gal4-TR, especially in the presence of 9-cis-RA. Liganded RXRα significantly inhibits T3-mediated activation by Gal4-TR, whereas liganded RXRβ shows little effect (Fig. 6). Since RXRα and -β share extensive similarities in their DBDs and LBDs, it seems likely that their unique N termini (the A/B domains) might be primarily responsible for the differential modulatory effects by these two receptors.

To test this possibility, we generated mutant versions of RXRα and -β (designated as RXRαΔA/B and RXRβΔA/B, respectively), where the entire A/B domain of each receptor is deleted. We then compared the effect of RXRαΔA/B and
RXRβΔA/B on Gal4-TR activity with that of full-length RXRα and RXRβ in transfection studies. Consistent with our earlier result, liganded full-length RXRα significantly inhibited the activity of Gal4-TR (by about 4-fold), whereas full-length RXRβ showed little inhibition (Fig. 9A). Interestingly, the two A/B domain deletion mutants, RXRαΔA/B and RXRβΔA/B, both exhibited little inhibition of Gal4-TR activity and were indistinguishable from each other in this assay (Fig. 9A). Thus, removal of their respective A/B domains abolishes the differential modulations of Gal4-TR activity originally exhibited by full-length RXRα and -β. This result indicates that the unique A/B domain of RXRα plays an important role in its inhibition of Gal4-TR activity and reveals a novel role for the N-terminal A/B domain in mediating the intermolecular cross-talk between partners of a receptor heterodimer.

We extended this study by examining the potential modulatory role of the RXRα A/B domain in a TRE-bound TR/RXR heterodimer. To this end, HeLa cells were transfected with TR and TRE-DR4A-CAT, together with either a control vector or a vector expressing either full-length hRXRα or hRXRβΔA/B. Consistent with our earlier results (see Fig. 7, A and B), cotransfection of full-length RXRα without 9-cis-RA inhibited TR-mediated activation, whereas the presence of both T3 and 9-cis-RA resulted in superactivation (Fig. 9B). Interestingly, under similar conditions, apo-RXRαΔA/B failed to inhibit TR-mediated activation (Fig. 9B). Nevertheless, cotransfection of RXRαΔA/B with TR still led to superactivation by T3 and 9-cis-RA (Fig. 9B). Thus, these results confirm that apo-RXRα requires its A/B domain for negative modulation of TR activity, whether or not the heterodimer is bound to TRE. In the presence of 9-cis-RA, liganded RXRα also requires its A/B domain to further down-regulate TR activity when the heterodimer is not TRE-bound (Fig. 9A), but the A/B domain is not essential for superactivation of a TRE-bound heterodimer (Fig. 9B).

Potential Mechanism(s) for Modulation of TR Activity by the RXRα A/B Domain—Our results have shown that inhibition of TR activity by RXRα is most prominent when the heterodimer is not TRE-bound and when 9-cis-RA is present (e.g. see Fig. 2A) and that this negative modulation requires the RXRα A/B domain (Fig. 9A). To explore potential mechanism(s) underlying the modulatory role of RXRα A/B, we considered several possibilities. First, a previous study from our laboratory showed that the DBD of RXR can recruit a corepressor PSF (30). Thus, one possibility is that the A/B domain of RXRα acts to facilitate or enhance the recruitment of PSF by the DBD. A second possibility is that the A/B domain of RXRα itself binds to a yet-to-be-identified corepressor. Finally, the A/B domain of RXRα may directly inhibit TR activity via an intermolecular dialog (e.g. by mediating a conformational change in the heterodimer).

To address these possibilities, we first examined whether cPSF, a dominant negative form of PSF, would relieve RXRα-mediated inhibition of TR activity. Since cPSF binds to the DBD but is devoid of repression function, its expression relieves PSF-mediated repression (30). However, we found that cotransfection of cPSF had no effect on RXRα-mediated inhibition over a variety doses of cPSF used (Fig. 10A) (data not shown). To test the second possibility, we asked whether coexpression of RXRα A/B domain in trans would titrate the putative corepressor and relieve the inhibition by full-length RXRα. As shown in Fig. 10B, cotransfection of RXRα A/B failed to relieve the inhibition mediated by full-length RXRα, even when a 5-fold excess of the RXRα A/B domain expression vector was used. Taken together, these results argue against the first two possibilities raised above and suggest that the A/B domain of RXRα probably acts via a more direct intermolecular dialog within the context of the TR/RXR heterodimer.
RXR and Its Ligand in the TR/RXR Heterodimer

A Novel and Dynamic Role for RXR in Modulating the Activity of Its Heterodimeric Partner TR—Whereas it is generally accepted that RXR in heterodimerizing with its partner TR plays a role in facilitating the binding of TR to certain TREs, the potential direct role of RXR in the transcriptional activity of the TR/RXR heterodimer is not clearly documented. The Gal4-TR system used in our experiments has enabled us to directly assess the potential contribution of RXR to the transcriptional activity of the TR/RXR heterodimer. An immediate modulatory effect of RXR on the activity of its heterodimeric partner TR. This study led to several novel and unexpected findings: 1) heterodimerization of apo-RXRs (in the absence of 9-cis-RA) with Gal4-TR inhibits T3-mediated transactivation; 2) the inhibition of Gal4-TR activity by RXR is further enhanced by 9-cis-RA; 3) two different RXR subtypes (α and β) differentially modulate the activity of Gal4-TR; and 4) the N-terminal A/B domains of RXRα and -β are largely responsible for their differential modulatory effects. We also tested whether the modulatory effects of RXRs observed in the Gal4-TR system can extend to a TRE-bound TR/RXR heterodimer. Our study showed that the effects observed with the Gal4-TR system are largely applicable to the TRE-bound TR/RXR heterodimer in that 1) the activity of a TRE-bound TR is also inhibited by apo-RXRs, 2) this inhibition also requires the A/B domain of RXRα, and 3) RXRβ (at least under the described transfection conditions) appears to have little effect on TR activity when assayed with either Gal4-TR or TRE-bound TR. One interesting exception is that 9-cis-RA appears to differentially affect T3-mediated transactivation from the Gal4-TR/RXRα (which is inhibited by 9-cis-RA dependent upon the RXRα A/B domain) and TRE-bound TR/RXRα (which is super-activated by 9-cis-RA independent of the RXRα A/B domain) heterodimers. Taken together, our results not only provide further support for our recent proposition that the RXR component in a TR/RXR heterodimer is not a silent partner (25) but also reveal novel aspects of regulation of the activity of the TR/RXR heterodimer by RXR and RXR ligand.

**DISCUSSION**

Many members of the type II nuclear receptor subfamily function as heterodimers with RXR (4). Heterodimerization with RXR usually is required for or facilitates the binding of these receptors to their cognate HREs. RXR can also play a direct role in transcriptional regulation mediated by permissive heterodimers, which allow for ligand binding and transactivation by RXR (4, 14). In contrast, certain RXR heterodimers, such as those involving TR, RAR, and VDR, are thought to be nonpermissive, where RXR is believed to be incapable of ligand binding due to the allosteric control of its partner receptor, and thus is said to be a silent partner (4, 14). However, a number of recent studies indicate that RXR can bind its ligand in the RAR/RXR heterodimer and mediate functional or biological consequences (19–24). In a recent report, we presented a series of functional studies, indicating that RXR is also not silent in the TR/RXR heterodimer (25). Taken together, these studies indicate that the silent partner model needs to be reconsidered and raise the possibility that RXR may play more than a subordinate role in those heterodimers that are previously thought to be nonpermissive.

Our recent study suggests that ligand binding by RXR promotes the dissociation of a corepressor(s) from its heterodimeric partner TR, presumably through an induced conformational change in TR (25). While it may serve to attenuate the repression function of apo-TR and/or to enhance the activation function of liganded TR, ligand binding by RXR does not appear to enable direct transactivation from the apo-TR/RXR heterodimer (18, 25). Thus, the transcriptional activation function of the TR/RXR heterodimer still appears to be dictated by the TR ligand T3. Nevertheless, the notion that RXR is not a silent partner prompted us to further examine the role(s) of RXR and/or its ligand in the functional activity of the TR/RXR heterodimer.

In this report, we extended our study in these areas. Using a Gal4-TR chimera and a Gal4-reporter, we examined the potential modulatory effect of RXR on the activity of its heterodimeric partner TR. This study led to several novel and unexpected findings: 1) heterodimerization of apo-RXRs (in the absence of 9-cis-RA) with Gal4-TR inhibits T3-mediated transactivation; 2) the inhibition of Gal4-TR activity by RXRα is further enhanced by 9-cis-RA; 3) two different RXR subtypes (α and β) differentially modulate the activity of Gal4-TR; and 4) the N-terminal A/B domains of RXRα and -β are largely responsible for their differential modulatory effects. We also tested whether the modulatory effects of RXRs observed in the Gal4-TR system can extend to a TRE-bound TR/RXR heterodimer. Our study showed that the effects observed with the Gal4-TR system are largely applicable to the TRE-bound TR/RXR heterodimer in that 1) the activity of a TRE-bound TR is also inhibited by apo-RXRs, 2) this inhibition also requires the A/B domain of RXRα, and 3) RXRβ (at least under the described transfection conditions) appears to have little effect on TR activity when assayed with either Gal4-TR or TRE-bound TR. One interesting exception is that 9-cis-RA appears to differentially affect T3-mediated transactivation from the Gal4-TR/RXRα (which is inhibited by 9-cis-RA dependent upon the RXRα A/B domain) and TRE-bound TR/RXRα (which is super-activated by 9-cis-RA independent of the RXRα A/B domain) heterodimers. Taken together, our results not only provide further support for our recent proposition that the RXR component in a TR/RXR heterodimer is not a silent partner (25) but also reveal novel aspects of regulation of the activity of the TR/RXR heterodimer by RXR and RXR ligand.

**Fig. 10.** A, inhibition of Gal4-TR activity by RXRα is not relieved by cPSF. HeLa cells were transfected using Geneporter 2, with the pMC110 reporter (200 ng) and the Gal4-TR expression vector (10 ng), together with either a control plasmid or a vector expressing RXRα (60 ng of each) to examine the inhibition of Gal4-TR activity by liganded RXRα. When indicated, a vector expressing cPSF or a control vector (100 ng of each) was cotransfected. The fold-inhibition was determined as in Fig. 2B. B, co-expression of the RXRα A/B domain does not relieve the inhibition of Gal4-TR activity by RXRα. HeLa cells were similarly transfected as in A, except that instead of using cPSF, a vector expressing RXRα A/B or its control vector (300 ng each) was used in cotransfection.
negative regulatory role for RXRα in the transcriptional activity of the TR/RXR heterodimer. Interestingly, in this respect, different subtypes of RXR (α versus β) appear to function differentially in their heterodimers with TR. Thus, whereas apo-RXRα down-regulates TR activity, apo-RXRβ is virtually neutral (at least under described transcription conditions) (Figs. 6 and 8), raising an interesting possibility that the transcriptional activity of the TR/RXR heterodimer in vivo is influenced by the composition of its RXR subtype.

Another surprising finding from our study is that 9-cis-RA enhances RXRα-mediated inhibition of Gal4-TR activity (Fig. 2A). Additional experiments suggested that the inhibition of Gal4-TR activity by liganded RXRα is not due to squelching per se (Figs. 2B and 3A), and that instead, it appears to be mediated through heterodimerization of these two receptors (Fig. 3). This result further supports our recent suggestion that the RXR component within the TR/RXR heterodimer can bind its ligand in cells. Interestingly, whereas liganded RXRα potently inhibits Gal4-TR activity, liganded RXRβ manifests only a minor effect (Fig. 6), again suggesting that different RXR subtypes can differentially influence the activity of the resulting TR/RXR heterodimers in cells.

A Novel Role for the N-terminal A/B Domain in Mediating Intermolecular Cross-talk between Partners of a Receptor Heterodimer—The precise mechanism(s) by which liganded RXRα down-regulates the activity of Gal4-TR is not yet clear. As discussed earlier, the inhibition is not due to squelching and appears to be mediated by heterodimerization between liganded RXRα and Gal4-TR (Figs. 2B and 3, A and B). Moreover, the aforementioned heterodimerization does not seem to interfere with DNA binding by the Gal4 TRDBD, since the constitutive activity of a GT(408)V chimera is not inhibited by liganded RXRα (Fig. 5). Indeed, selective inhibition of the activity of Gal4-TR (which is due to the activation function of liganded TR) but not that of GT(408)V (which is due to the activation function of VP16) by liganded RXRα suggests that the inhibition is mediated by a specific intermolecular cross-talk between TR and RXR.

What might be the nature of this intermolecular cross-talk? Given the fact that only liganded RXRs but not β potently down-regulates Gal4-TR activity (Fig. 6), any proposed mechanism(s) needs to account for the differential effects manifested by RXRα and -β. Since the two RXRs share extensive similarity in their DBDs and LBDs and mainly differ in their N termini, it seems likely that the unique A/B domain of each receptor is primarily responsible for their differential modulation of Gal4-TR activity. Indeed, removal of the A/B domain abolishes the differential modulation and renders the two resulting mutant RXRs indistinguishable (Fig. 9A). The two ΔA/B mutants show little inhibition of Gal4-TR activity, which is reminiscent of the effect by RXRβ (Fig. 9A). Thus, our result indicates that the N-terminal A/B domain of RXRα is critical for its inhibition of Gal4-TR activity and reveals a novel role for the A/B domain in mediating certain intermolecular cross-talk between partners of a receptor heterodimer.

To further explore the mechanism(s) underlying the modulatory role of the RXRα A/B domain within the TR/RXR heterodimer, we considered several possibilities. A previous study from our group showed that the DBD of RXR can recruit a novel corepressor PSF (30). Thus, one possibility is that the RXRα A/B domain facilitates or enhances the recruitment of PSF by the DBD. Alternatively, the A/B domain of RXRα may recruit a yet-to-be-identified corepressor itself. However, cotransfection experiments using the dominant negative version of PSF and that using the RXRα A/B domain argue against these two possibilities (Fig. 10). Thus, we propose that the RXRα A/B domain may act via a more direct intermolecular dialog with TR (e.g. by mediating a conformational change of the heterodimer that would influence the binding and/or dissociation of a coactivator(s) and/or corepressor(s)). This kind of allosteric control between heterodimeric receptor partners is well documented (17, 25, 41).

A Novel Role for HRE Binding in Determining the Effect of an RXR Ligand—Two aspects of the modulation of TR activity by RXRα observed with the Gal4-TR system seem also applicable to a TRE-bound TR/RXR heterodimer: 1) apo-RXRα also negatively modulates TR activity in the context of a TRE-bound heterodimer (Fig. 7A), and 2) the negative modulation by RXRα is dependent on its A/B domain (Fig. 9B). One interesting exception is that 9-cis-RA differentially modulates the activities of the Gal4-TR/RXRα and TRE-bound TR/RXRα heterodimers. Thus, T3-mediated transactivation from a Gal4-TR/RXRα heterodimer is inhibited by 9-cis-RA, whereas treatment with both T3 and 9-cis-RA superactivates the TRE-bound TR/RXRα heterodimer (Fig. 7B). This result suggests that a TR/RXR heterodimer bound or not bound to a TRE responds differentially to 9-cis-RA and thus reveals a novel role for HRE binding in determining the effect of an RXR ligand. Interestingly, previous studies with GR have documented that the nature of GRES can modify the transcriptional activity of liganded GR via an allosteric control conferred by the GRE sequence (42). In this regard, our finding that HRE binding switches the effect of a ligand in an RXR heterodimer (from negative to positive) adds a novel scheme to the regulation of receptor activities by HREs.

The mechanism by which HRE binding switches the effect of 9-cis-RA in the TR/RXR heterodimer is not clear. It is plausible that HRE binding exerts an allosteric control (42) that relieves the inhibition on TR activity imposed by liganded RXRα when the heterodimer is “idle” (not bound to a TRE). The relief of inhibition would then enable liganded RXRα to augment TR activity by facilitating the dissociation of a corepressor(s) and/or promoting the association of a coactivator(s) (25). An intriguing implication of our finding is that an idle TR/RXRα heterodimer occupied by ligands would be kept in a state of relatively low “activity” until it is bound to a TRE, which may provide a novel layer of regulation for the activities of receptors and their ligands.

Heterodimerization Integrates Multiple Informational Inputs—Heterodimerization with RXR is a common strategy employed by many type II receptors to exert their biological functions (4). A well known role for heterodimerization is to facilitate the binding of receptors to their HREs. In this report, we carried out detailed functional analysis of the TR/RXR heterodimer. Our results not only provide further support to our recent study that RXR is not a silent partner in the TR/RXR heterodimer (25) but also reveal several novel functional aspects of receptor heterodimerization. Our results suggest that different subtypes of RXRs, as well as the presence or absence of the RXR ligand, differentially modulate the activity of the TR/RXR heterodimer (Figs. 1, 2A, 6, 7A, and 8). In addition, HRE binding or lack of differentially affects the effect of 9-cis-RA on the activity of a TR/RXRα heterodimer (Fig. 7B). Thus, in addition to its well known role in facilitating DNA binding by the receptors, heterodimerization can function in integrating multiple informational inputs (such as presence or absence of a ligand or ligands, partner receptor subtypes, HRE binding, or lack of) to dynamically modulate receptor activities.

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