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The Ligand-Binding Domains of the Thyroid Hormone/Retinoid Receptor Gene Subfamily Function In Vivo To Mediate Heterodimerization, Gene Silencing, and Transactivation

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The ligand-binding domains (LBDs) of the thyroid/retinoid receptor gene subfamily contain a series of heptad motifs important for dimeric interactions. This subfamily includes thyroid hormone receptors (T3Rs), all-*trans* retinoic acid (RA) receptors (RARs), 9-*cis* RA receptors (RARs and retinoid X receptors [RXRs]), the 1,25-dihydroxyvitamin D_3 receptor (VDR), and the receptors that modulate the peroxisomal β-oxidation pathway (PPARs). These receptors bind to their DNA response elements in vitro as heterodimers with the RXRs. Unliganded receptors in vivo, in particular the T3Rs, can mediate gene silencing and ligand converts these receptors into a transcriptionally active form. The in vivo interactions of these receptors with RXR were studied by using a GAL4-RXR chimera containing the yeast GAL4 DNA-binding domain and the LBD of RXRβ. GAL4-RXR activates transcription from GAL4 response elements in the presence of 9-*cis* RA. Unliganded T3R, which does not bind or activate GAL4 elements, represses the activation of GAL4-RXR by 9-*cis* RA in HeLa cells. However, addition of T3 alone leads to transcriptional activation. These findings suggest that T3R can repress or activate transcription while tethered to the LBD of GAL4-RXR and that heterodimerization can occur in vivo without stabilization by hormone response elements. Similar ligand-dependent activation was observed in HeLa cells expressing RAR, VDR, or PPAR and in GH4C1 cells from endogenous receptors. Replacement of the last 17 amino acids of the LBD of RXRβ with the 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein leads to a GAL4 constitutive activator that is repressed by wild-type T3R but not by a ninth heptad mutant that does not form heterodimers. This finding suggests that the ninth heptad of T3R is important for gene silencing and that the LBD of RXR does not exhibit silencing activity. This conclusion was verified with GAL4-LBD chimeras and with wild-type receptors in assays using appropriate response elements. These studies indicate that the LBD has diverse functional roles in gene regulation.

Thyroid hormone (T3) receptors (T3Rs), all-*trans* retinoic acid (RA) receptors (RARs), and 9-*cis* RA receptors (RARs and retinoid X receptors [RXRs]) are part of a subfamily within the steroid receptor superfamily (12, 19, 22). Other members of this subfamily include the 1,25-dihydroxyvitamin D_3 (Vit D) receptor (VDR) (2, 32, 46), receptors which mediate the peroxisomal proliferation response to fatty acids and other factors (PPARs) (18, 33, 36, 38), and several orphan receptors for which ligands are either unknown or not required for activity (e.g., the COUP-TFs) (14, 61). Unlike the steroid receptors for glucocorticoids, progestins, mineralocorticoids, and estrogens, which bind their DNA response elements as homodimers (15, 30, 42), T3Rs, RARs, RXRs, VDR, and the PPARs bind to their DNA elements as homodimers (1, 22, 26, 60, 66) and/or as heterodimers (e.g., T3R/RXR, RAR/RXR, T3R/RAR, VDR/RXR, and PPAR/RXR) (1, 10, 21, 28, 37, 39, 41, 43, 45, 63, 65). Heterodimerization among these receptors involves the conserved heptad repeat region within their ligand-binding domains (LBDs). These interactions between RXR and T3R or RXR and RAR can occur in the absence of ligands or DNA (17, 45) but may be stabilized by DNA binding in vitro (21).

The ninth heptad is the most highly conserved of the heptad repeats among this subfamily of receptors, and mutations in the ninth heptad region eliminate the interaction of T3R or RAR with RXR (1, 48). Mutants of chicken T3Rα (cT3Rα) (408 amino acids) or human RARα (hRARα) (462 amino acids) with changes in the first hydrophobic amino acid of the ninth heptad [cT3Rα(L365R) or hRARα(M377R)] form homodimers on their response elements with or without ligand but form heterodimers with RXR only in the presence of T3 or RA (1). This suggests that the ninth heptads of T3R or RAR mediate heterodimerization in the absence of ligand and that binding of T3 or RA exposes a different region in the LBDs of T3R or RAR which then interacts with RXR (1).

In addition to the effects mentioned above, the ninth heptad of the T3R LBD appears to be critical for two effects of T3R: dominant negative inhibition by the T3R LBD (1, 48) and gene silencing of active promoters containing response elements by unliganded wild-type T3R (1). Dominant negative inhibition by the T3R LBD, which involves heterodimerization and requires an intact ninth heptad, could occur by several mechanisms. One mechanism involves formation of nonfunctional cT3Rα-LBD/RXR heterodimers in solution which would sequester RXR and decrease its availability to form T3R/RXR or RAR/RXR heterodimers which then bind DNA. An alternative mechanism involves disruption of T3R/RXR or RAR/

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RXR heterodimers already bound to DNA. In contrast to its ability to inhibit wild-type T3R or RAR (21, 55), the LBD of cT3Rα enhances activation by two yeast GAL4-receptor chimeras (13). One chimera, GAL4-T3R, contains the DNA-binding domain of the yeast GAL4 protein linked in frame to the entire LBD of cT3Rα (amino acids 120 to 408). This GAL4-T3R activates a GAL4-responsive reporter gene in the presence of T3 (13). The second chimera, GAL4-T3R-VP16, is expressed as a protein containing the GAL4 DNA-binding domain, amino acids 120 to 392 of cT3Rα, followed by the 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein (13, 57). Since GAL4-T3R-VP16 lacks the last 16 amino acids of the LBD of cT3Rα, the chimera poorly binds T3 and is not activated by ligand (13).

Although GAL4-VP16 is a potent transcriptional activator, GAL4-T3R-VP16 is inactive (13). Surprisingly, coexpression of the LBD of cT3Rα converts GAL4-T3R-VP16 into a potent ligand-independent activator and paradoxically enhances T3-mediated activation by GAL4-T3R. Activation of GAL4-T3R-VP16 and GAL4-T3R by the LBD of cT3Rα appears to result from competition for a transcriptional inhibitor which interacts with the ninth heptad regions of these chimeric receptors. These divergent effects of the LBD of cT3Rα are explained by differences in the DNA-binding properties of wild-type and chimeric GAL4-receptors (13). GAL4 binds to its response elements as a dimer through a dimerization domain in its DNA-binding domain (11). Thus, unlike wild-type T3R or RARs, which bind to DNA as heterodimers that are stabilized through their LBDs, the LBD of cT3Rα would not block the binding of GAL4-receptor chimeras to DNA. Because of this difference in DNA binding, GAL4-receptor chimeras are useful probes for revealing aspects of protein-protein interactions and gene regulation that would not be possible with wild-type receptors containing their cognate DNA-binding domains. In this study, we used GAL4-RXR and GAL4-RXR-VP16 chimeras as probes of DNA-independent interactions between RXR and related members of this receptor subfamily. These studies provide new insights into the nature of heterodimeric interactions of RXR with T3R, RAR, VDR, and PPAR and related factors in mediating ligand-dependent transcriptional activation and the active silencing of genes by unliganded receptor.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells, Rat-2 fibroblasts cells, and GH4C1 rat pituitary cells were cultured and transfected by electroporation as previously described (1, 20, 24, 55). The chloramphenicol acetyltransferase (CAT) reporter vectors and other plasmids used are described below. After incubation for 48 h with or without the indicated ligand(s), cells were harvested and CAT activity was measured by using a thin-layer chromatography assay (20, 24). Acetylated and unreacted [^{14}C]chloramphenicol was excised from the thin-layer plate and quantitated in a liquid scintillation counter. The amount of protein used in the assays was adjusted to keep the conversion of [^{14}C]chloramphenicol within the linear range below 40%. CAT activity values were normalized to represent the percentage of [^{14}C]chloramphenicol acetylated by a specific amount of cell protein in 16 h at 37°C. All experiments were performed with duplicate or triplicate flasks and were repeated at least three times with essentially identical results. The results presented in each figure or table represent transfections that were performed in the same experiment.

Plasmids. Plasmids expressing full-length cT3Rα and deletion mutants cT3Rα(1-392) and cT3Rα(120-408) have been previously described (21, 24, 55). cT3Rα(120-408) contains the entire LBD and binds T3 with an affinity similar to that of wild-type cT3Rα (31). The affinity of cT3Rα(1-392) for T3 is too low to measure accurately and is at least 50-fold less than that of wild-type receptor (31). cT3Rα(L365R) contains the indicated single amino acid change in the ninth heptad of the LBD of cT3Rα (1). Rat T3Rα1 (rT3Rα1) and rat c-ErbAα2 (r-c-ErbAα2) (40) were cloned into a pEXPRESS (pEX) vector (1, 23). hRARα(M377R) (1) contains the indicated single amino acid change in the ninth heptad of the LBD of hRARα. hRARα(155-462) expresses the entire LBD of hRARα (13). hRARα(1-403) was constructed from wild-type hRARα by cleavage of the 3′ end of hRARα cDNA with *Sma*I and cloning the cDNA into a Rous sarcoma virus (RSV) long terminal repeat (LTR) expression vector (pRSV) (24). Receptor expression vectors for cT3Rα, hRARα, and mouse RXRβ (mRXRβ) proteins were regulated by the RSV LTR in either pRSV (24) or pEX (23). A hPPARγ cDNA encoding a 478-amino-acid protein was cloned in the laboratory of one of the authors (M. E. Greene) and will be described in detail elsewhere. hPPARγ was expressed by using a pcDNAI/Amp vector (Invitrogen). A pRc/CMV vector (Invitrogen) expressing hVDR was obtained from Leonard Freedman (26).

GAL4-VP16 (53), pSG424 (53), and pMC110 (53), a GAL4-regulated CAT reporter, were obtained from Mark Ptashne. A GAL4 chimera containing regions of mRXRβ was constructed from pSG424, which expresses the DNA-binding domain of GAL4 from simian virus 40 (SV40) early promoter sequences and contains a polylinker sequence downstream of the region encoding the GAL4 DNA-binding domain (amino acids 1 to 147) (54). GAL4-RXR contains the entire LBD from amino acids 185 to 438 (41) of mRXRβ (29) linked in frame to the GAL4 DNA-binding domain. The nucleotide sequences corresponding to amino acids 185 to 438 in mRXRβ were amplified by PCR with the introduction of an *Eco*RI site at both 5′ and 3′ ends. The PCR products were cleaved with *Eco*RI and cloned into the *Eco*RI site of the pSG424 polylinker. GAL4-RXR-VP16 contains amino acids 1 to 147 of GAL4 followed by amino acids 185 to 421 of mRXRβ and the 90-amino-acid transactivation domain of the herpes simplex virus VP16 protein (57). The nucleotide sequences corresponding to amino acids 185 to 421 in mRXRβ were also amplified by PCR with the introduction of an *Eco*RI site at both 5′ and 3′ ends. The PCR products were cleaved with *Eco*RI and cloned into the *Eco*RI site in the polylinker of GAL4-VP16. Orientations were determined by restriction enzyme analysis. GAL4-T3R is a pSG424-based construct containing the entire LBD (amino acids 120 to 408) of cT3Rα linked in frame to the GAL4 DNA-binding domain (13). GAL4-r-c-ErbAα2 was constructed by replacing the *Nco*I-*Kpn*I fragment of GAL4-T3R with that of r-c-ErbAα2. pRSVT7 vectors were constructed for use in GH4C1 cells and for experiments using SV40 promoter-based CAT vectors. pRSVT7 is identical to pRSV (24), except that it contains a bacteriophage T7 promoter sequence cloned into the *Hin*dIII site downstream of the RSV LTR. GAL4-T3R (13) and pSG424 (54) were digested with *Hin*dIII and *Acc*65I, and these fragments were cloned into pRSVT7 to create RSVT7-GAL4-T3R and RSVT7-GAL4-DBD. RSVT7-GAL4-T3R(L365R) was constructed by replacing the *Sac*I-*Sal*I fragment of RSVT7-GAL4-T3R with that of cT3Rα(L365R) (1). RSVT7-GAL4-RXR was constructed by inserting the *Hin*dIII-*Xba*I fragment of GAL4-RXR into pRSVT7.

ΔMTV-TREp-CAT contains one copy of the palindromic thyroid hormone response (TREp) element (AGGTCA TGACCT) with flanking *Hin*dIII-cohesive ends (21) cloned into the *Hin*dIII site at position −88 of ΔMTV-CAT (58). Similarly, ΔMTV-RDR+5-CAT contains one copy of the retinoid-responsive RDR+5 element (59) cloned into ΔMTV-CAT. ΔSV-TREp-CAT contains one copy of the TREp inserted at position −135 of the enhancerless SV40 early promoter (59). pG5-SV-BCAT contains five GAL4-responsive binding sites cloned upstream of the SV40 early promoter linked to the CAT gene (62).

RESULTS

The LBDs of T3R and RAR are dominant negative inhibitors of RXR. The LBD of cT3Rα [cT3Rα(120-408)] inhibits transcriptional activation by wild-type T3R and RAR in a dominant negative fashion in several cell lines, including HeLa and GH4C1 (21, 24). HeLa cells express very low levels of T3Rs and RARs (21), while rat pituitary GH4C1 cells express receptors for T3, RA, and 9-*cis* RA that regulate expression of the endogenous rat growth hormone gene (7, 24, 27). Mutations in the first or last hydrophobic amino acid of the ninth heptad of cT3Rα or hRARα [cT3Rα(L365R), cT3Rα(L372R), hRARα(M377R), and hRARα(L384R)] eliminate dominant negative activity in vivo and heterodimerization with mRXRβ in vitro (1). Although wild-type cT3Rα and hRARα bind as heterodimers with RXR with or without ligands, cT3Rα (L365R) and hRARα(M377R) form heterodimers with RXR only in the presence of their cognate ligands, T3 and RA. Consistent with these in vitro findings, these receptor mutants mediate dominant negative activity only in the presence of ligand, while wild-type receptors mediate inhibition with or without ligand (1). This finding suggests that the inhibitory effect of cT3Rα(120-408) on wild-type cT3Rα or hRARα results from the heterodimerization of cT3Rα(120-408) with RXR, which is endogenously expressed in HeLa cells (41), that

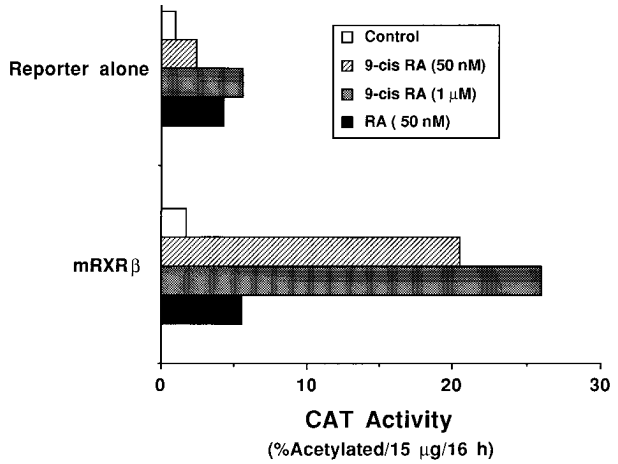

FIG. 1. Activity of mRXRβ in Rat-2 cells. Rat-2 cells were transfected by electroporation with 5 μg of ΔMTV-TREp-CAT alone or with 10 μg of a vector expressing mRXRβ. Following transfection, cells were incubated for 48 h without or with 50 nM 9-*cis* RA, 1 μM 9-*cis* RA, or 50 nM RA prior to determination of CAT activity.

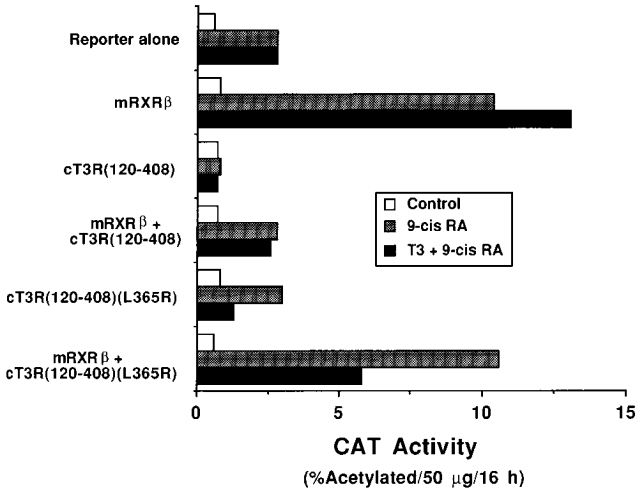

FIG. 2. cT3Rα(120-408) and cT3Rα(120-408)(L365R) mediate different effects on mRXRβ activity. Rat-2 cells were transfected by electroporation with 5 μg of pMC110 alone or with the following amounts of expression vectors: 10 μg of mRXRβ, 10 μg of mRXRβ plus 2 μg of cT3Rα(120-408), 10 μg of mRXRβ plus 6 μg of cT3Rα(120-408)(L365R), 2 μg of cT3Rα(120-408), or 6 μg of cT3Rα(120-408)(L365R). Following transfection, cells were incubated for 48 h without ligand or with 1 μM 9-*cis* RA or with 1 μM T3 plus 1 μM 9-*cis* RA prior to determination of CAT activity.

blocks the formation of functional RXR/cT3Rα or RXR/hRARα heterodimers.

Studies using Rat-2 fibroblasts, a cell line that expresses low levels of RXR, RAR, and T3R, provide further evidence for the possible functional importance of RXR heterodimers. Expression of mRXRβ in Rat-2 cells strongly enhances the 9-*cis* RA-dependent activation of the retinoid-responsive reporters ΔMTV-TREp-CAT (Fig. 1) and ΔMTV-RDR+5-CAT (data not shown). This enhancement reflects ligand-dependent activation by mRXRβ and not enhancement of endogenous RAR activity by transfected RXR, since very little stimulation was mediated by RA (Fig. 1). cT3Rα(120-408) inhibits 9-*cis* RA-dependent stimulation by endogenous RXR-like activity and mRXRβ (Fig. 2) and by hRXRα (data not shown). The LBD of mutant cT3Rα(L365R) [cT3Rα(120-408)(L365R)], which requires T3 for interaction with RXR in vitro (1), inhibits endogenous RXR-like activity and mRXRβ only with T3 (Fig. 2). Although this inhibition is only partial, this effect of T3 with the cT3Rα(120-408)(L365R) mutant on mRXRβ activity was highly reproducible. The LBD of hRARα [hRARα(155-462)] also acts as a dominant inhibitor of mRXRβ and RXR-like activity in Rat-2 cells (Fig. 3). These findings are consistent with the notion that the LBDs of T3R or RAR inhibit wild-type T3R or RAR by competing for RXR (1), thus blocking the formation of active wild-type RXR/T3R or RXR/RAR heterodimers. This inhibition may be mediated by the disruption of DNA-bound heterodimers or by the sequestration of RXR, resulting in decreased availability of RXR to bind hormone response elements as active heterodimers with T3R or RAR. Consistent with the latter model is the finding that mRXRβ can form heterodimers with T3R or RAR in vitro without binding to DNA (17, 45).

In vivo interactions between the LBDs of RXR and T3R, RAR, VDR, or PPAR do not require binding to hormone response elements. A GAL4-RXR chimera was used to determine whether the LBD of RXR could form heterodimers in vivo with T3R, RAR, VDR, and PPAR without stabilization by their cognate hormone response elements. This chimera consists of the yeast GAL4 DNA-binding domain linked in frame to the LBD (amino acids 185 to 438) of mRXRβ. The use of GAL4-receptor chimeras has several advantages for studying functional interactions of T3R, RAR, PPAR, or VDR with RXR. First, these wild-type receptors do not bind or activate the yeast GAL4 response element (13). Thus, inhibitory effects of one receptor on the activity of another member of the thyroid/retinoid receptor subfamily do not result from competition for hormone response elements with overlapping specificity. Second, GAL4 binds to its response element as a dimer through a region in its DNA-binding domain (11). Consequently, the LBDs of T3R or RAR, which inhibit dimerization of wild-type receptors (21, 55), do not inhibit the dimeric binding of GAL4 chimeras to DNA. Thus, inhibition or stimulation of the activity of GAL4-receptor chimeras by wild-type

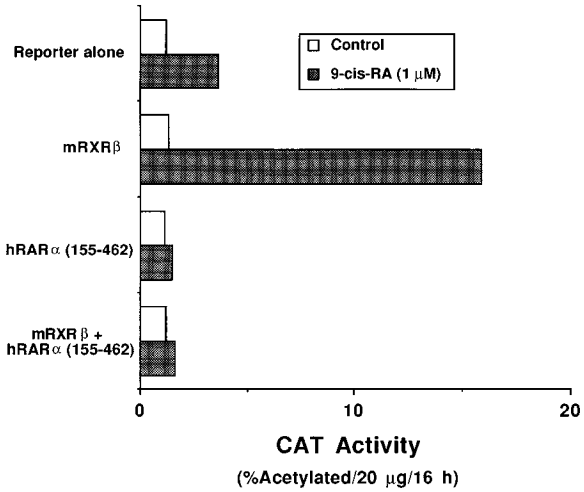

FIG. 3. Dominant negative effects of hRARα(155-462) on mRXRβ. Rat-2 cells were transfected by electroporation with 5 μg of ΔMTV-TREp-CAT alone or with 10 μg of mRXRβ, 10 μg of mRXRβ plus 5 μg of hRARα(155-462), or 5 μg of hRARα(155-462) expression vectors. Following transfection, cells were incubated for 48 h without or with 1 μM 9-*cis* RA prior to determination of CAT activity.

TABLE 1. Effect of T3 on the functional interaction between T3R and GAL4-RXR[a]

Transfection	Amt (μg)	CAT activity (% acetylated/20 μg of protein/16 h)			
		Basal	T3	9-*cis* RA	T3 + 9-*cis* RA
pMC110	5	0.1	0.1	0.1	0.1
+GAL4-RXR	1	0.1	0.1	37.4	38.8
+cT3Rα	3	0.1	5.2[b]	4.4	9.4
+cT3Rα(L365R)	6	0.2	5.4[c]	34.9	29.3
+cT3Rα(1-392)	4	0.1	0.1	0.6	0.6
+rT3Rα1	4	0.1	5.3	2.5	5.4
+r-c-ErbAα2	4	0.2	0.3	39.7	40.7

[a] HeLa cells were transfected by electroporation with the indicated amounts of pMC110 alone or together with expression vectors as specified. Following transfection, cells were incubated for 48 h without (basal) or with 1 μM T3, 1 μM 9-*cis* RA, or both ligands prior to determination of CAT activity.
[b] With 20 nM T3, CAT activity was 4.6.
[c] With 20 nM T3, CAT activity was 1.3.

receptors or their LBDs occurs by mechanisms independent of DNA binding.

In the following studies, control experiments indicated that the T3Rs, hRARα, hVDR, or hPPARγ did not influence the activity of pMC110, a GAL4-responsive reporter gene, when expressed alone, with the LBD of mRXRβ, or with the GAL4 DNA-binding domain (reference 13 and data not shown). Expression of GAL4-RXR leads to 9-*cis* RA-dependent activation of pMC110 (Tables 1 and 2). Coexpression of cT3Rα, rT3Rα1 (Table 1), or cT3Rα(120-408) (data not shown) with GAL4-RXR resulted in T3-dependent activation. cT3Rα(1-392) (24) and r-c-ErbAα2 (40) have low affinities for T3 and do not mediate T3-dependent activation when coexpressed with GAL4-RXR (Table 1). cT3Rα(L365R), which forms heterodimers with RXR only in the presence of T3 in gel mobility assays (1), also mediates T3-dependent activation with GAL4-RXR. These findings suggest that T3R can activate transcription while bound to the LBD of GAL4-RXR and that heterodimerization occurs in vivo without concomitant binding to specific hormone response elements. This conclusion is supported by a recent gel shift study using a GAL4 response element, the results of which indicated that RXRβ can form a heterodimer with GAL4-rT3Rα1 (amino acids 121 to 410) (35). In the absence of T3, cT3Rα, rT3Rα1, and cT3Rα(1-392) act to inhibit GAL4-RXR activity in the presence of 9-*cis* RA (Table 1). Since these receptors do not inhibit the activity of

TABLE 2. Functional interactions between RAR, VDR, or hPPARγ and GAL4-RXR[a]

Transfection	Amt (μg)	CAT activity (% acetylated/40 μg of protein/16 h)				
		Basal	RA	Vit D	WY	9-*cis* RA
GAL4-RXR	1	0.2	0.9	0.3	0.2	59.9
+hRARα	4	0.4	39.5			52.3
+hRARα(M377R)	4	0.5	29.6			62.6
+hRARα(1-403)	4	0.6	0.5			1.6
+hVDR	2	0.3		27.2		44.8
+hPPARγ	4	0.6			13.8	61.5

[a] HeLa cells were transfected by electroporation with 5 μg of pMC110 and expression vectors as specified. Following transfection, cells were incubated for 48 h without (basal) or with 100 nM RA, 100 nM Vit D, 100 μM WY 14,643 (WY), or 1 μM 9-*cis* RA prior to determination of CAT activity.

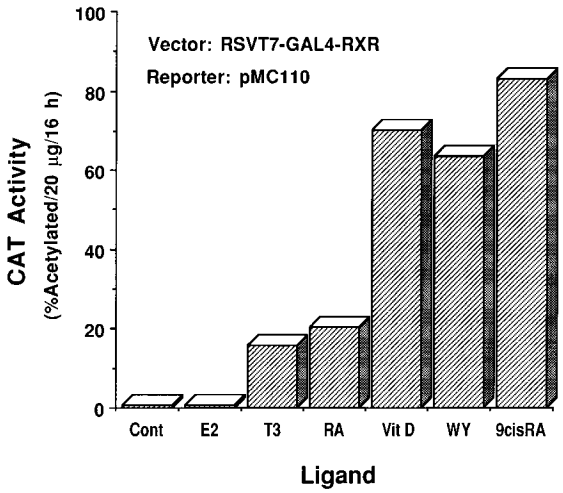

FIG. 4. Activation of GAL4-RXR by endogenous levels of T3R, RAR, VDR, or PPAR. GH4C1 cells were transfected by electroporation with 5 μg of pMC110 along with 1 μg of the pRSVT7 vector expressing GAL4-RXR. Following transfection, cells were incubated for 48 h without (control [Cont]) or with 100 nM estradiol (E2), 100 nM T3, 50 nM RA, 100 nM Vit D, 100 μM WY 14,643 (WY), or 1 μM 9-*cis* RA prior to determination of CAT activity.

GAL4-VP16 (13), they appear to act by dimerizing with the LBD of RXR and not by preventing binding of the GAL4 domain to its DNA response element. This conclusion is supported by the finding that unliganded cT3Rα(L365R) and r-c-ErbAα2, which do not heterodimerize with RXR (1, 34, 35), do not inhibit 9-*cis* RA-dependent GAL4-RXR activity (Table 1).

Coexpression of hRARα, hVDR, or hPPARγ with GAL4-RXR results in RA-, Vit D-, or WY 14,643-dependent activation of a GAL4-responsive reporter (Table 2). GAL4-RXR alone was not activated by any of these ligands. hRARα(1-403), which retains the ninth heptad but does not activate ΔMTV-TREp-CAT in the presence of RA or 9-*cis* RA (data not shown), did not activate in the presence of RA (Table 2). hRARα(M377R), which forms heterodimers with RXR only in the presence of RA in gel mobility assays (1), also mediated RA-dependent activation with GAL4-RXR (Table 2). These findings suggest that T3, RA, Vit D, and WY 14,643 mediate ligand-dependent activation when these receptors heterodimerize with the LBD of GAL4-RXR. Furthermore, the weak inhibition of GAL4-RXR activity by VDR and the strong inhibition by hRARα(1-403) indicate that, like T3R, these receptors can interact in vivo with the LBD of RXR in the absence of their cognate ligands.

Endogenous T3Rs, RARs, VDRs, and PPARs mediate activation through DNA-independent heterodimerization with the LBD of GAL4-RXR. GH4C1 pituitary cells express endogenous receptors for T3, RA, Vit D, and estradiol (about 10,000 molecules of each receptor per cell) and are responsive to physiological concentrations of these ligands (7, 24, 25, 47, 50). Since the transient expression methods used in the experiments described in Tables 1 and 2 may lead to significantly elevated levels of receptor expression, studies were also performed with GH4C1 cells to determine whether receptors at endogenous levels could mediate ligand-dependent activation while bound to the LBD of GAL4-RXR. Figure 4 shows that when GH4C1 cells express GAL4-RXR, the GAL4 reporter pMC110 is stimulated by T3, RA, Vit D, and WY 14,643, indicating that GH4C1 cells also express PPAR. Thus, even at endogenous concentrations, the receptors for these ligands

TABLE 3. T3 reverses the inhibition of GAL4-RXR-VP16 by T3R[a]

Transfection	Amt (μg)	CAT activity (% acetylated/20 μg of protein/16 h)		
		Basal	T3	9-*cis* RA
GAL4-RXR-VP16	1	35.6	30.1	35.4
+cT3Rα	2	2.9	30.0	3.8
+cT3Rα(L365R)	6	42.7	36.5	42.8
+cT3Rα(1-392)	2	0.8	0.8	1.2
+rT3Rα1	4	1.8	18.9	4.3
+r-c-ErbAα2	4	39.6	40.2	37.3
+cT3Rα(120-408)	2	7.1	75.1	7.5

[a] HeLa cells were transfected by electroporation with 5 μg of pMC110 and expression vectors as specified. Following transfection, cells were incubated for 48 h without (basal) or with 1 μM T3 or 1 μM 9-*cis* RA prior to determination of CAT activity.

interact with RXR without stabilization by their cognate response elements. As expected, estradiol did not activate through GAL4-RXR (Fig. 4) since the estrogen receptor does not interact with RXR in vitro (63).

Activation by GAL4-RXR-VP16 is repressed by unliganded wild-type receptors. GAL4-RXR-VP16 is a chimera that contains amino acids 185 to 421 of mRXRβ inserted between the GAL4 DNA-binding domain and the C-terminal 90-amino-acid transactivating domain of the herpes simplex virus VP16 protein (57). The region inserted from the LBD of RXR is analogous to amino acids 120 to 392 in the LBD of cT3Rα. We found previously that GAL4-T3R-VP16, which contains amino acids 120 to 392 of cT3Rα, is inactive alone but is activated by coexpression with unliganded T3R or RAR but not RXR (13). These and other studies suggested that an inhibitory factor represses the activity of GAL4-T3R-VP16 by interacting with the ninth heptad region of the T3R LBD (13). Coexpressed T3R or RAR or their LBDs effectively compete for this inhibitory factor and relieve the repression of GAL4-T3R-VP16. Coexpressed RXR does not lead to activation of GAL4-T3R-VP16, suggesting that it might not interact with the inhibitory factor.

Unlike GAL4-T3R-VP16 (13) or GAL4-RAR-VP16 containing amino acids 155 to 404 of hRARα (data not shown), GAL4-RXR-VP16 is a constitutive activator of the GAL4-responsive pMC110 reporter (Tables 3 and 4). This result further suggests that RXR may not interact with the inhibitory factor believed to repress the activity of the corresponding T3R and RAR chimeras (13). 9-*cis* RA did not affect the activity of GAL4-RXR-VP16 (Table 3), suggesting that the last 17 amino acids of the LBD of mRXRβ may affect 9-*cis* RA binding or its ability to convert the protein into a transcriptionally active form. cT3Rα and rT3Rα1 inhibited the constitutive activity of GAL4-RXR-VP16 without or with 9-*cis* RA, and this inhibition was reversed by T3. Unliganded cT3Rα(120-408) also inhibited the constitutive activity of GAL4-RXR-VP16. However, T3 reversed this inhibition and further enhanced transcriptional activation above the level observed with GAL4-RXR-VP16 alone (Table 3). This enhanced activation suggests that the reversal of repression by T3 is not caused by dissociation of a heterodimer between the unliganded LBD region of T3R and GAL4-RXR-VP16 that inhibits the chimera but rather is caused by T3-mediated activation of the heterodimeric complex similar to that found with GAL4-RXR and T3Rs (Table 1). In contrast, cT3Rα(L365R) and r-c-ErbAα2 did not inhibit GAL4-RXR-VP16 with or without 9-*cis* RA. This result also suggests that repression is caused by a direct interaction of the LBD of T3R with RXR since cT3Rα(L365R) and r-c-ErbAα2 do not interact with RXR (1, 34, 35). hVDR also repressed GAL4-RXR-VP16, while hRARα, hRARα(1-403), and hPPARγ did not mediate repression (Table 4). This finding suggests that the interaction of hRARα and hPPARγ with the RXR-VP16 moiety may mediate different conformational changes compared with cT3Rα, rT3Rα1, and hVDR or that hRARα and hPPARγ lack intrinsic silencing or inhibitory activity. However, hVDR and hRARα do mediate ligand-dependent enhancement of the basal activity of GAL4-RXR-VP16 (Table 4), while hPPARγ showed little or no enhancement in the presence of WY 14,643.

TABLE 4. Functional interactions between hRARα, hVDR, or hPPARγ and GAL4-RXR-VP16[a]

Transfection	Amt (μg)	CAT activity (% acetylated/30 μg of protein/16 h)				
		Basal	RA	Vit D	WY	9-*cis* RA
GAL4-RXR-VP16	1	27.0	26.7	26.8	23.8	31.0
+hRARα	5	23.2	60.1			30.9
+hRARα(1-403)	5	24.5	25.0			26.2
+hVDR	1	7.8		24.4		7.6
+hPPARγ	4	29.3			31.9	32.0

[a] HeLa cells were transfected by electroporation with 5 μg of pMC110 and expression vectors as specified. Following transfection, cells were incubated for 48 h without (basal) or with 100 nM RA, 100 nM Vit D, 100 μM WY 14,643 (WY), or 1 μM 9-*cis* RA prior to determination of CAT activity.

RXR does not mediate silencing of gene expression. The finding that GAL4-RXR-VP16 is constitutively active and that cT3Rα represses this activity is reminiscent of the silencing activity of unliganded T3R (1, 4, 5, 8, 16). Previous studies with active basal promoters containing TREs have shown that unliganded T3R can suppress basal activity and that T3 acts both to reverse the suppression and to increase activity above basal levels (1, 8, 16). Silencing is dependent on the ninth heptad region, since cT3Rα(L365R) did not mediate silencing but showed normal stimulatory activity with T3 (1). Thus, the results of Table 3 suggest that repression occurs when unliganded T3R, which exhibits silencing activity, associates with the LBD of RXR.

The relative repression or silencing activities of the LBDs of cT3Rα, cT3Rα(L365R), and mRXRβ were compared by linking the entire LBDs of these receptors (amino acids 120 to 408 for the cT3Rα proteins and 185 to 438 for mRXRβ) to the GAL4 DNA-binding domain. These constructs were expressed from a vector regulated by the RSV LTR that does not alter the activity of the SV40 viral early promoter of the pG5-SV-BCAT reporter, which contains five GAL4 response elements. In HeLa cells, the basal activity of pG5-SV-BCAT was repressed by GAL4-T3R but not by the GAL4 DNA-binding domain (GAL4-DBD), GAL4-T3R(L365R), GAL4–r-ErbAα2, or GAL4-RXR (Table 5, experiment 1). All of the chimeras which bind ligand mediate similar levels of ligand-dependent activation. Unlike wild-type cT3Rα, unliganded mRXRβ did not repress the basal activity of ΔSV-TREp-CAT in Rat-2 cells (Table 5, experiment 2). As previously described (1), cT3Rα(L365R) also did not repress the basal activity of ΔSV-TREp-CAT (Table 5, experiment 2). Despite these differences in silencing activity, cT3Rα, cT3Rα(L365R), and mRXRβ all mediate similar levels of ligand-dependent activation of ΔSV-TREp-CAT. mRXRβ (Fig. 1) and hRXRα (data not shown) also do not suppress basal gene activity of ΔMTV-TREp-CAT or ΔMTV-RDR+5-CAT (data not shown) in Rat-2 cells, but

TABLE 5. cT3Rα but not mRXRβ suppresses basal gene expression through a mechanism requiring its ninth heptad[a]

Transfection	Amt (μg)	CAT activity (% acetylated/20 of μg/16 h)		
		Basal	T3	9-*cis* RA
Expt 1				
G5-SV-BCAT	1	48.2	48.0	48.7
+GAL4-DBD	1	47.3		
+GAL4-T3R	1	16.1	240	
+GAL4-T3R(L365R)	1	49.4	194	
+GAL4-r-c-ErbAα2	1	47.3	47.8	
+GAL4-RXR	1	44.3		149
Expt 2				
ΔSV-TREp-CAT	5	6.9	7.0	18.7
+cT3Rα	2	1.9	65.3	
+cT3Rα(L365R)	6	6.8	60.1	
+mRXRβ	10	7.6		55.3

[a] In experiment 1, HeLa cells were transfected by electroporation with G5-SV-BCAT alone or together with expression vectors as indicated. GAL4-DBD, GAL4 DNA-binding domain (amino acids 1 to 147) expressed by pRSVT7. GAL4-r-c-ErbAα2 was expressed by using 1 μg of a pSG424-based expression vector. In experiment 2, Rat-2 cells were transfected by electroporation with ΔSV-TREp-CAT alone or together with expression vectors as indicated. Following transfection, Rat-2 cells or HeLa cells were incubated for 48 h without (basal) or with 1 μM T3 or 1 μM 9-*cis*-RA prior to determination of CAT activity.

both stimulate gene expression with 9-*cis* RA. These results suggest that RXR does not possess an active silencing domain within its LBD and that the ninth heptad of T3R is required for TRE-mediated silencing of basal gene activity by unliganded T3R.

DISCUSSION

Heterodimerization of T3R, RAR, VDR, and PPAR with the LBD of RXR can occur in vivo without binding to hormone response elements. Although many in vitro studies have documented heterodimerization between RXR and T3R, RAR, VDR, and PPAR (1, 10, 21, 28, 36–39, 41, 43–45, 56, 63, 65), few studies provide evidence that these heterodimeric interactions occur in vivo. The most compelling evidence comes from studies showing that mutant receptors that bind DNA in vitro as homodimers but not as heterodimers with RXR do not mediate dominant negative activity (1, 48). In addition, Nagpal et al. (49) reported that RA could activate transcription of a GAL4 reporter gene in cells transfected with a GAL4-RXR construct and a plasmid expressing wild-type hRARα, hRARβ, or hRARγ. Although this study suggested that heterodimerization of hRAR could occur with the LBD of RXR "in solution" in vivo, this conclusion was complicated by two issues. First, these studies were carried out with pSG5 expression vectors in Cos-1 cells, which leads to plasmid amplification and extremely high levels of protein expression. Second, the reporter contained a GAL4 DNA-binding sequence adjacent to an estrogen receptor response element (ERE). Since hRARα is capable of binding to and activating the ERE in the context of certain promoters (21), this study could not exclude the possibility that heterodimerization occurred between GAL4-RXR and hRAR when bound to the adjacent GAL4 and ERE sequences.

To provide additional evidence for heterodimerization in vivo, we examined functional interactions of GAL4-RXR with the endogenous T3R, RAR, PPAR, and VDR in GH4C1 cells and with these same receptors expressed in HeLa cells, which provide lower levels of receptor expression than Cos-1 cells (51). These studies show that when GAL4-RXR is expressed, each of these receptors can activate pMC110, a GAL4 reporter gene, in the presence of its cognate ligand (Tables 1 and 2; Fig. 4). These findings suggest that T3R, RAR, PPAR, and VDR can activate transcription while tethered to the LBD of GAL4-RXR and that heterodimerization can occur in vivo without stabilization by hormone response elements. Our observation that T3-occupied cT3Rα(120-408), which lacks a DNA-binding domain, also activates GAL4-RXR further supports this conclusion. Consistent with the finding that RXR does not form heterodimers in vitro with the estrogen receptor (63), estradiol did not mediate activation through the endogenous estrogen receptor in GH4C1 cells.

Heterodimerization in vivo can occur independently of ligand binding and leads to diverse biologic effects. The finding that ligand-occupied T3R, RAR, PPAR, and VDR activate through GAL4-RXR does not establish whether these receptors heterodimerize with RXR in the absence of their ligands in vivo or whether ligand enhances heterodimerization. In vitro binding studies indicate that heterodimerization of wild-type cT3Rα or hRARα with RXR is not dependent on the presence of T3 or RA (1). In contrast, receptors with mutations in the ninth heptad, cT3Rα(L365R) and hRARα(M377R), form heterodimers with RXR only in the presence of T3 or RA (1). This finding suggests that the region of the ninth heptad of T3R or RAR is involved in heterodimerization with RXR in the absence of ligand, while a ligand-mediated conformational change exposes another region of T3R or RAR that interacts with RXR (1). Support for this conclusion comes from studies showing that heterodimerization of VDR with RXR can occur in vitro without ligand (63) but is markedly increased by Vit D (43, 44, 56).

Our results provide further evidence that wild-type T3R can interact with the LBD of RXR in vivo in the absence of ligand, while the L365R heptad mutant requires T3 for this interaction. Unliganded cT3Rα and rT3Rα1 inhibit the 9-*cis* RA activation of GAL4-RXR, while unliganded cT3Rα(L365R) and r-c-ErbAα2, which do not heterodimerize with RXR, do not inhibit activation by 9-*cis* RA (Table 1). Deletion of the final 16 amino acids of cT3Rα in cT3Rα(1-392) markedly reduces T3 binding and eliminates a putative transactivation domain but not the heterodimerization domain (6, 52, 64). cT3Rα(1-392) retained the ability to inhibit activation by 9-*cis* RA but did not mediate T3 activation of GAL4-RXR, confirming the importance of heterodimerization for the inhibitory activity (Table 1). Similarly, cT3Rα, cT3Rα(1-392), and cT3Rα(120-408) inhibited constitutive activation by GAL-RXR-VP16, while the activity of this chimera was not reduced by cT3Rα(L365R) or r-c-ErbAα2 (Table 3).

hPPARγ did not suppress activation of GAL4-RXR-VP16 (Table 4) although it activated through GAL4-RXR in the presence of its synthetic ligand (Table 2). If unliganded PPAR forms heterodimers with RXR in vivo as previously shown in vitro (36, 38), then its interaction with RXR must be functionally different from that of T3R, which did suppress activation by GAL4-RXR-VP16. Since both RAR and RXR are activated by 9-*cis* RA, it is not possible to examine directly whether hRARα can interfere with the activation of RXR (Table 2). However, hRARα(1-403), which is not activated by 9-*cis* RA, blocked the activation of GAL4-RXR by ligand, suggesting that the interaction of RAR with RXR could influence the activation properties of RXR (Table 2). In contrast, hRARα(1-403) did not suppress GAL4-RXR-VP16 activity, although hRARα appears capable of interacting with GAL4-RXR-VP16 since expression of hRARα leads to activation by RA (Table 4). These findings imply that heterodimerization of hRARα(1-403) with RXR can constrain an intrinsic activation

domain in the LBD of RXR whereas it does not repress the transactivation domain of VP16. Although hVDR expression led to activation by Vit D, unliganded hVDR partially suppressed the 9-*cis* RA-mediated activation of GAL4-RXR (Table 2). Similarly, unliganded hVDR suppressed the constitutive activity of GAL4-RXR-VP16 (Table 4). Although our studies do not indicate whether ligand enhances the interaction of VDR with RXR, they clearly document that VDR can interact in vivo with RXR in the absence of Vit D.

The LBD of T3R but not RXR contains an active silencing function which requires the conserved ninth heptad. The finding that T3R can repress activation by both GAL4-RXR and GAL4-RXR-VP16 is reminiscent of the active repression or silencing of basal gene expression by unliganded T3R (1, 4, 5, 8, 16). This silencing has been suggested to result from the interaction of DNA-bound unliganded T3R with transcription factors (e.g., TFIIB) which are important for basal gene transcription (3). This repression may result from a direct interaction of T3R with a basal transcription factor or an indirect interaction mediated by a bridging protein which links the receptor to the transcription factor. Our studies indicate that only GAL4-T3R acts to repress the basal expression of pG5-SV-BCAT, while GAL4-DBD, GAL4-T3R(L365R), GAL4–r-c-ErbAα2, and GAL4-RXR do not repress even though ligand-dependent activation occurred with their cognate ligands (Table 5, experiment 1).

The findings presented above suggest that the LBD of RXR does not contain a silencing function and that the ninth heptad of T3R is important for active silencing. This is consistent with a previous study which showed that cT3Rα(L365R) does not result in active silencing and that ligand-dependent transcriptional activation is not simply the reversal of silencing of basal expression mediated by unliganded cT3Rα (1). However, the failure of cT3Rα(L365R) to mediate silencing in this earlier study could have resulted from a reduction of DNA binding, since this mutant does not bind to its response element as a heterodimer with RXR in the absence of T3 (1). Since GAL4-T3R does not appear to require RXR to bind to GAL4 elements (4, 35), the finding that GAL4-T3R(L365R) does not repress basal gene activity suggests that the region of the ninth heptad is important in mediating silencing independent of binding to RXR, perhaps by directly interacting with a basal transcription factor or indirectly via a protein other than RXR. The finding that GAL4-RXR does not mediate silencing suggests that if RXR enhances the silencing effect of wild-type T3R, it does so by enhancing the DNA binding of T3R rather than by contributing an intrinsic silencing function to the heterodimer. Additional evidence for this conclusion comes from studies using wild-type cT3Rα, cT3Rα(L365R), and mRXRβ with ΔSV-TREp-CAT in Rat-2 cells, which contain low levels of endogenous RXR (Table 5, experiment 2). These results indicate that gene silencing is mediated by wild-type cT3Rα but not by cT3Rα(L365R) or mRXRβ, although these two receptors mediate similar levels of ligand-dependent stimulation.

Implications for receptor function in vivo. Members of the thyroid/retinoid receptor gene family bind as heterodimers to diverse hormone response elements that are organized as direct, inverted, or everted repeats separated by different-size-nucleotide gaps (9, 59). The mechanisms of response element recognition are poorly understood. An analysis of the homodimeric binding of cT3Rα to the inverted repeat (TREp) sequence, AGGTCA TGACCT, suggested that cT3Rα homodimers do not sequentially interact with each half-site but that the receptor forms metastable homodimers in solution which then bind the response element, which stabilizes the protein-protein interaction (21). Our results suggest a similar model for the DNA binding of heterodimers containing RXR and members of the thyroid/retinoid receptor gene family, since we find that T3R, RAR, VDR, and PPAR can interact with RXR in vivo independent of binding to their DNA elements. These heterodimers would then bind to their cognate response elements, where their interactions may be further stabilized by DNA binding. Formation of heterodimers independent of DNA could allow for the existence of a variety of subtle conformational differences which could permit the recognition of both direct, inverted, or everted repeat sequences.

The role of ligand in the formation and/or DNA binding of heterodimers in vivo is currently unclear, although 9-*cis* RA has been reported to favor the in vitro formation of RXR homodimers over that of heterodimers containing RXR (66). Activation of transcription by ligands such as RA or T3 is presumed to be mediated by conformation changes in their cognate receptors leading to the formation or exposure of a transactivating domain. GAL4-T3R mediates levels of T3-mediated transcriptional activation similar to those of the heterodimer-bound wild-type T3R on reporter genes containing the mouse mammary tumor virus LTR (pMC110 and ΔMTV-TREp-CAT, respectively) (this study and references 5 and 13). This finding raises the possibility that RXR primarily acts to enhance the effect of wild-type T3R by increasing DNA binding of T3R rather than by contributing an independent activation function to the heterodimer. Furthermore, RXR in the T3R/RXR heterodimer does not appear to contribute to the silencing activity found with unliganded T3R. Evidence to support this view comes from our studies indicating that neither GAL4-RXR nor wild-type RXR exhibits silencing activity, while GAL4-T3R and wild-type T3R efficiently repress basal gene expression (Table 5). These differences may explain why GAL4-RXR-VP16 is constitutively active and repressed by unliganded wild-type T3R (Table 3) whereas GAL4-T3R-VP16 is inactive but can be activated by coexpressing wild-type T3R or its LBD in the absence of T3 (13). This activation of GAL4-T3R-VP16 appears to result from the titration of an inhibitory factor which associates with the T3R LBD of the DNA-bound chimera (13). Dissociation of the inhibitor from the T3R LBD also appears to be mediated by T3 (13). This inhibitor, which associates with the ninth heptad region of T3R and may not efficiently interact with RXR (13), could play an important role in the gene-silencing activity mediated by unliganded T3R.

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