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Unique Response Pathways Are Established by Allosteric Interactions among Nuclear Hormone Receptors

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Summary

Heterodimerization is a common paradigm among eukaryotic transcription factors. The 9-cis retinoic acid receptor (RXR) serves as a common heterodimerization partner for several nuclear receptors, including the thyroid hormone receptor (T_3R) and retinoic acid receptor (RAR). This raises the question as to whether these complexes possess dual hormonal responsiveness. We devised a strategy to examine the transcriptional properties of each receptor individually or when tethered to a heterodimeric partner. We find that the intrinsic binding properties of RXR are masked in T_3R–RXR and RAR–RXR heterodimers. In contrast, RXR is active as a non-DNA-binding cofactor with the NGFI-B/Nurr1 orphan receptors. Heterodimerization of RXR with constitutively active NGFI-B/Nurr1 creates a novel hormone-dependent complex. These findings suggest that allosteric interactions among heterodimers create complexes with unique properties. We suggest that allostery is a critical feature underlying the generation of diversity in hormone response networks.

Introduction

Heterodimerization is a common theme in eukaryotic regulatory biology. A number of transcription factor families have been defined by their characteristic dimerization interface. These include the leucine zipper (e.g., Fos, Jun, cAMP response element–binding protein, and CCAAT enhancer–binding protein) (Lamb and McKnight, 1991), helix-loop-helix (e.g., Myc, Max, MyoD, E12, and E47) (Amati and Land, 1994), Rel (NF-κB and dorsal) (Blank et al., 1992), ankyrin (GA-binding protein) (Brown and McKnight, 1992), and the nuclear receptor superfamilies (Evans, 1988; Forman and Samuels, 1990). Detailed analysis of these proteins have shown that heterodimerization produces novel complexes that bind DNA with higher affinity or altered specificity (Glass, 1994). In contrast, little is known about the contributions of each monomer toward the transcriptional properties of the complex. Nuclear receptor heterodimers provide an ideal system to examine these questions since transcriptional responses are regulated by steroids, retinoids, thyroid hormones, fatty acids, and other signaling molecules (Evans, 1988).

Nuclear hormone receptors are characterized by a central DNA-binding domain (DBD) (Figure 1A), which targets the receptor to specific DNA sequences known as hormone response elements (HREs). The all-*trans* retinoic acid receptor (RAR), the receptor of the thyroid hormone receptor (T_3R), the vitamin D3 receptor (VDR), and the fatty acid/peroxisome proliferator–activated receptor (PPAR) preferentially bind to DNA as heterodimers with a common partner, the 9-cis retinoic acid X receptor (RXR) (Yu et al., 1991; Bugge et al., 1992; Kliewer et al., 1992a; Leid et al., 1992; Marks et al., 1992; Zhang et al., 1992; Issemann et al., 1993). Naturally occurring HREs are composed of direct repeats (DRs) (Umesono et al., 1991), inverted repeats (IRs) (Umesono et al., 1988; Williams et al., 1991), or everted repeats (ERs) (Baniahmad et al., 1990; Farsetti et al., 1992; Raisher et al., 1992; Tini et al., 1993) of a degenerate X_n-AGGTCA core site. The DBD contains two helical regions, one of which serves as a recognition helix that makes base-specific contacts within the major groove of the core site (Luisi et al., 1991; Schwabe et al., 1993). A third helix has been identified in some receptors that makes additional minor groove contacts in the 5′ portion of the core-binding site (X_n) (Wilson et al., 1992; Lee et al., 1993). In DRs (head-to-tail arrangement), the X_n sequence also serves as a gap that separates the two core-binding sites. Spacers of 1, 3, 4, and 5 nt serve as preferred response elements for heterodimers of RXR with PPAR, VDR, T_3R, and RAR, respectively (Naar et al., 1991; Umesono et al., 1991; Kliewer et al., 1992b; Issemann et al., 1993). The optimal gap length for each heterodimer is determined by protein–protein contacts that appropriately position the DBDs of RXR and its partner (Kurokawa et al., 1993; Perlmann et al., 1993; Towers et al., 1993; Predki et al., 1994; Zechel et al., 1994). In contrast with this mode of DNA binding, a growing number of receptor-like proteins have been identified that bind as a monomer to a single core site. The orphan receptors of the NGFI-B (also known as nur77, N10, NAK-1, and TR3) and Nurr1 (also known as RNR-1, NOT, and HZF-3) family provide well-characterized examples of this paradigm (Wilson et al., 1993).

Once bound to an HRE, each receptor responds to its signal through the C-terminal ligand-binding domain (LBD) that binds its cognate hormone with high affinity and specificity (Evans, 1988; Forman and Samuels, 1990). The LBD is a complex entity containing several embedded subdomains. These include a C-terminal transactivation function (AF-2), a series of heptad repeats that serve as a dimerization interface, and a poorly delineated transcriptional suppression domain (Figure 1A) (Forman and Samuels, 1990). AF-2 resides at the C-terminus (Zenke et al., 1990; Danielian et al., 1992; Nagpal et al., 1993; Durand et al., 1994) and consists of approximately 20 amino acids with the potential to form an amphipathic α helix (Barettino et al., 1994; Schulman et al., submitted). When linked to a heterologous DBD, AF-2 displays constitutive transcriptional activity (Barettino et al., 1994; Schulman et al., submitted). However, in the natural context of the LBD, transcriptional activity requires the addition of ligand. This indicates that the LBD functions as a modular unit whose transcriptional

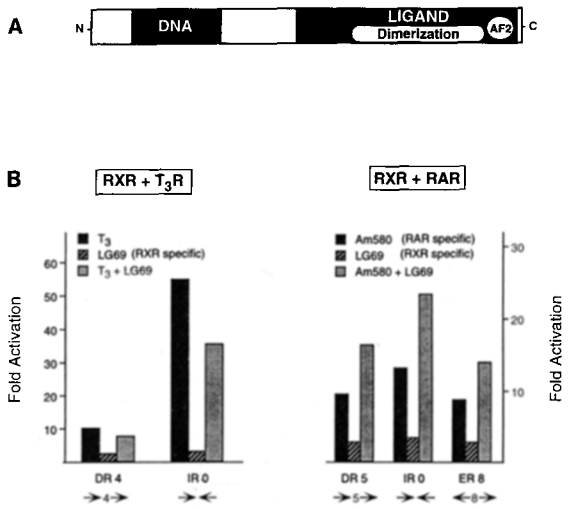

Figure 1. RXR Responsiveness Is Differentially Modulated by T_3R and RAR

(A) Functional domains of nuclear hormone receptors. The location of key functional domains in nuclear receptors are shown schematically. The box marked DNA represents the DBD. The box marked ligand represents the large C-terminal LBD. Dimerization and transactivation (AF-2) functions are embedded within this region.

(B) Transient transfection analysis of T_3R–RXR and RAR–RXR heterodimers. Reporter constructs contain the indicated HRE cloned upstream of the TK–LUC reporter. In the experiment shown on the left, CV-1 cells were transfected with the following plasmids: HRE × 2 TK–LUC (300 ng/10^5 cells), CMX–h$T_3R\beta$ (20 ng/10^5 cells), CMX–hRXRα (20 ng/10^5 cells), and the internal control CMX–β-gal (500 ng/10^5 cells). Cells were treated without ligand or with 100 nM T_3, 100 nM LG69, or 100 nM T_3 and 100 nM LG69. In the experiment shown on the right, cells were transfected with HRE × 1 TK–LUC (300 ng/10^5 cells), CMX–hRARα (50 ng/10^5 cells), CMX–hRXRα (50 ng/10^5 cells), and CMX–β-gal (500 ng/10^5 cells). Cells were treated without ligand or with 50 nM Am580, 100 nM LG69, or 50 nM Am580 and 100 nM LG69. Normalized LUC activity was determined and plotted as fold activation relative to untreated cells.

activities are controlled by ligand. Thus, it may be assumed that both members of a receptor heterodimer will be simultaneously activated by their specific ligands. Contrary to this expectation, we now provide evidence that the ligand-induced transcriptional activities of RXR are suppressed when complexed with RAR and T_3R. This suppression occurs at the levels of ligand binding and transcriptional activation and can be further modulated by the ligand for the partner. Furthermore, RXR responsiveness was not observed with other partners, including VDR.

As part of a search for additional RXR partners, we were surprised to find that the RXR LBD can interact productively with Nurr1, a member of the nuclear receptor superfamily that binds DNA as a monomer (Law et al., 1992; Scearce et al., 1993). As a result of this interaction, the constitutive activity of Nurr1 is suppressed, and the complex becomes 9-cis RA dependent. Thus, an LBD can create a hormone-dependent complex even though it is not directly bound to DNA. These unique properties establish the Nurr1–RXR complex as a novel response pathway for RXR signals.

Taken together, our findings suggest that heterodimer formation imparts allosteric changes upon the LBD of nuclear receptors. These allosteric changes confer transcriptional activities onto the heterodimer that are distinct from those of the component monomers. This arrangement permits a limited number of regulatory proteins to generate a diverse set of transcriptional responses to multiple hormonal signals.

Results

RXR Responsiveness Is Diminished in T_3R–RXR and RAR–RXR Heterodimers

Since T_3R and RAR function as heterodimers with RXR, we sought to examine RXR responsiveness in the context of T_3R–RXR and RAR–RXR heterodimers. Although cells transfected with both $T_3R\beta$ and RXRα expression vectors were responsive to T_3, they were surprisingly not responsive to the RXR-specific ligand LG69 (Figure 1B) (Boehm et al., 1994). Treatment of these cells with both T_3 and LG69 did not result in further stimulation of the T_3 response; instead, the response to T_3 was somewhat reduced (Figure 1B) (Hallenbeck et al., 1993; Lehmann et al., 1993). Similarly, cells simultaneously transfected with RARα and RXRα expression vectors responded to the RAR-specific ligand Am580 but remained unresponsive to LG69 (Figure 1B). In contrast, treatment with Am580 and LG69 resulted in increased transcriptional activity over that seen with Am580 alone (Figure 1B).

Suppression of RXR Activity Is Mediated by the LBD

Since RXR homodimers are activated by RXR selective agonists (Mangelsdorf et al., 1991; Lehmann et al., 1992), our results suggest that RXR activity is suppressed in unliganded T_3R–RXR and RAR–RXR heterodimers. We speculated that heterodimerization within the LBD (Figure 1A) could induce an allosteric change in the RXR LBD that blocks its ability to bind ligand, to transactivate, or both. To test this hypothesis, we developed a system that examines the responsiveness of RXR-containing heterodimers in a manner that relies solely on interactions between the LBDs. A chimeric protein was constructed containing the yeast GAL4 DBD linked to the RXR LBD (GAL–RXR). The ability of this RXR chimera to respond to LG69 was initially examined in the presence of truncated receptors containing the LBDs of T_3R or RAR. Although GAL–RXR activated the upstream activating sequence (UAS_G) reporter in response to LG69, the absolute levels of induced and uninduced activity were dramatically suppressed by both T_3R and RAR LBDs (Figure 2A). In contrast, the VDR LBD failed to suppress RXR responsiveness. These results indicate that suppression of RXR by unliganded T_3R and RAR is mediated solely by interactions between the LBDs of these receptors.

Our results are consistent with previous experiments that have shown that receptor LBDs remain tethered to the GAL–RXR LBD in cells (Nagpal et al., 1993). Thus, we asked whether the tethered LBDs can activate transcription in response to their specific ligands. As seen in Figure 2A, the T_3R, RAR, and VDR LBDs conferred

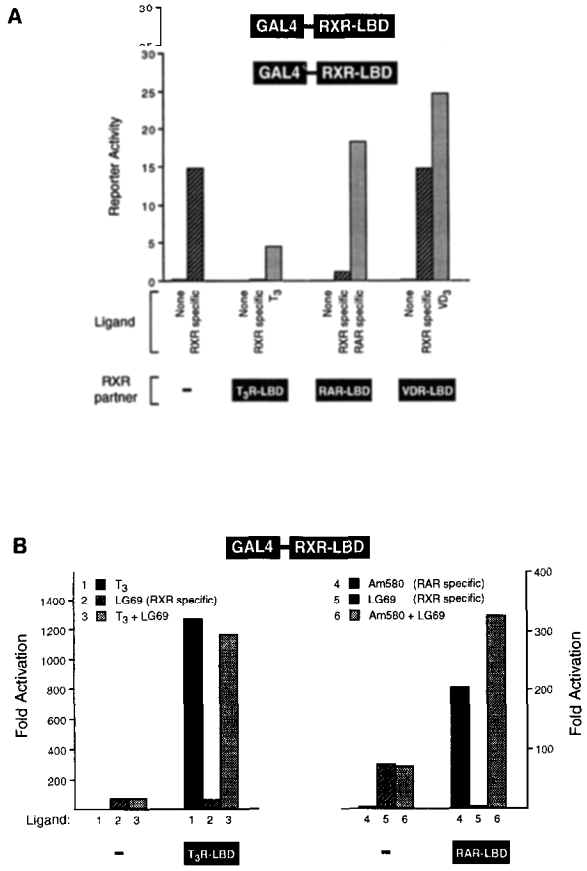

Figure 2. Transcriptional Activity of RXR is Differentially Modulated by the LBDs of T_3R and RAR

(A) Transient transfection analysis of GAL–RXR LBD in the presence of T_3R, RAR, or VDR LBDs. Reporter constructs contained four copies of the UAS_G cloned upstream of the TK–LUC reporter. CV-1 cells were transfected with UAS_G × 4 TK–LUC (300 ng/10^5 cells), CMX–GAL–RXR (100 ng/10^5 cells), or CMX–β-gal (500 ng/10^5 cells) alone or with either CMX–T_3R LBD, CMX–RAR LBD, or CMX–VDR LBD (100 ng/10^5 cells). Following transfection, cells were treated without ligand or with 100 nM LG69, 100 nM T_3, 50 nM Am580, or 100 nM VD_3. Normalized LUC activity was determined and plotted as reporter activity.

(B) The cells in (A) were additionally treated with 100 nM T_3 and 100 nM LG69 (left) or 50 nM Am580 and 100 nM LG69 (right). Normalized LUC activity was determined and plotted as fold activation relative to untreated cells.

ligand-dependent activation upon GAL–RXR, but not on GAL4 alone (data not shown). Thus, receptor LBDs tethered to RXR provide all the functions required for ligand-dependent transcriptional activation in the absence of direct DNA contact.

The experiment of Figure 2A was also performed with the combination of RXR-specific (LG69) and T_3R- or RAR-specific ligands (Figure 2B). To compare the effects of T_3R and RAR LBDs on LG69 inducibility of GAL–RXR, these data were replotted as fold induction. Comparison of Figures 1B and 2B indicates that the effects of ligand-occupied T_3R and RAR are qualitatively similar whether the full-length receptors or their LBDs are used. Note that the T_3R LBD led to a coordinate reduction in both basal and LG69-induced activities of GAL–RXR (Figure 2A); hence,

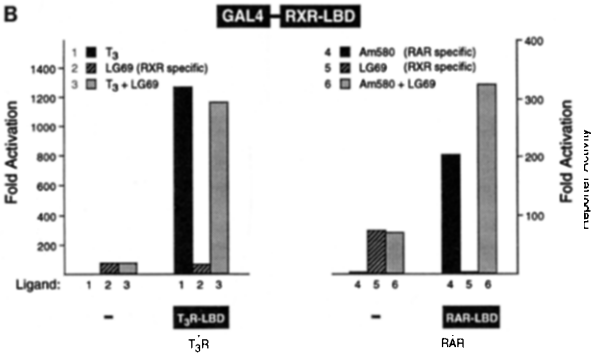

Figure 3. T_3R and RAR Suppress Transcription of a Constitutively Active RXR Derivative

In the experiment shown on the left, CV-1 cells were transfected with the following plasmids: with IR0 TK–LUC (300 ng/10^5 cells) or CMX–β-gal (500 ng/10^5 cells) alone (minus) or with CMX–VP16–RXRα (100 ng/10^5 cells), CMX–hT_3Rβ (50 ng/10^5 cells), or both, as indicated. In a separate experiment, shown on the right, cells were transfected with IR0 TK–LUC (300 ng/10^5 cells) or CMX–β-gal (500 ng/10^5 cells) alone (minus) or with CMX–VP16–RXRα (100 ng/10^5 cells), CMX–hRARα (50 ng/10^5 cells), or both, as indicated. No ligand treatment was employed. LUC activity was normalized to the β-gal internal control. In each experiment, the normalized activity obtained in the presence of VP16–RXR, T_3R, or RAR is plotted as activity relative to the reporter alone, which was defined to have a relative activity of 1.

the fold response to LG69 was only modestly inhibited from 69-fold (GAL–RXR alone; Figure 2B, left) to 57-fold by the T_3R LBD (GAL–RXR and T_3R LBD; Figure 2B, left). Addition of T_3 resulted in strong activation of T_3R, and the combination of T_3 and LG69 resulted in slightly less activity than with T_3 alone. In contrast with T_3R, unliganded RAR LBD strongly suppressed the fold responsiveness of GAL–RXR to LG69. Treatment with Am580 and LG69 resulted in increased transcriptional activity over that seen with Am580 alone, suggesting that RXR responsiveness to LG69 may be partially restored by addition of the RAR agonist Am580 (Figure 2B).

T_3R and RAR Suppress Activation of RXR-Containing Heterodimers

Taken together, our results indicate that T_3R and RAR act in *trans* to suppress the absolute transcriptional activity of RXR. Ligand-free RAR additionally suppresses the ability of RXR to activate in response to its ligand. In contrast, ligand-free T_3R only modestly suppresses RXR inducibility; full suppression requires the addition of T_3R ligands, suggesting that ligand binding by one partner can affect the activity of the other.

When bound to DNA, unliganded T_3R and RAR are known to suppress basal transcription (Damm et al., 1989; Baniahmad et al., 1992). This is thought to occur by direct interaction of the LBD with the basal transcription factor TFIIB (Baniahmad et al., 1993; Fondell et al., 1993). We speculated that, in addition to basal suppression, T_3R and RAR may also interfere with TFIIB-dependent activation. To test this idea, we fused the potent herpes VP16 *trans*-activation domain to the N-terminus of RXR (VP16–RXR). As expected from previous studies (Underhill et al., 1994), VP16 confers ligand-independent activity onto the VP16–RXR chimera (Figure 3). Since VP16 activates transcription by recruiting TFIIB into the preinitiation complex (Rob-

erts and Green, 1994), the constitutive activity of VP16–RXR is presumed to represent TFIIB-dependent activation. In cotransfection experiments, unliganded T_3R and RAR inhibited VP16–RXR activation of the IR0 reporter (Figure 3). Suppression was observed on other RXR response elements, including cellular retinol-binding protein II (CRBPII) (DR1) and an ER separated by 8 nt (data not shown), indicating that suppression occurs independent of specific HRE architecture. These data indicate that unliganded T_3R and RAR suppress TFIIB-dependent activation when complexed with RXR.

RAR and T_3R Differentially Suppress the Ligand-Binding Activity of RXR

In addition to decreasing basal and activated transcription, RAR also blocked the ability of RXR to respond to its ligand (see Figure 2B). Thus, we examined the possibility that RXR was incapable of binding ligand when tethered to RAR. A bacterially expressed glutathione S-transferase–RXRα fusion protein (GST–RXR) was incubated with recombinant T_3R or RAR in the presence of radiolabeled RXR ligands. The amount of ligand bound to RXR or RXR-containing heterodimers was quantitated using glutathione–Sepharose as an affinity probe. As expected, binding of [^{3}H]LG69 to GST–RXR was specifically competed by unlabeled LG69, but not by the RAR-specific ligand Am580 (Figure 4A, left). Conversely, GST–RXR was incapable of binding to [^{3}H]all-*trans* RA (Figure 4A, right); specific binding of [^{3}H]all-*trans* RA was observed when GST–RXR was mixed with excess RAR (Figure 4A, right). A quantitation of the amount of specifically bound [^{3}H]LG69, [^{3}H]all-*trans* RA, or [125I]T_3 (data not shown) indicated that GST–RXR could be saturated with approximately equimolar amounts of RAR or T_3R, respectively (see legend to Figure 4; data not shown). Electrophoretic mobility shift experiments indicated that ligands did not alter the binding activity of T_3R–RXR or RAR–RXR heterodimers (data not shown).

Next, we examined the ligand-binding activity of RXR in the presence of RAR or T_3R. Surprisingly, addition of RAR resulted in a dramatic (>85%) decrease in the amount of [^{3}H]LG69 bound to GST–RXR (Figure 4B), indicating that the ligand binding potential of RXR is reduced in the RAR–RXR heterodimer. These findings account for the ability of unoccupied RAR to suppress the ligand inducibility of RXR (see Figure 2B, right).

Similar experiments were performed on the T_3R–RXR heterodimer. In contrast with RAR, unliganded T_3R led to a modest reduction in [^{3}H]LG69 binding. However, ligand binding was strongly diminished upon addition of T_3 (Figure 4B). These findings are consistent with the observation that unoccupied T_3R results in a modest suppression of RXR inducibility, whereas no further induction is elicited when T_3R is occupied by T_3 (see Figure 2B, left).

The transfection experiments in Figures 1B and 2B indicate that RAR–RXR heterodimers exhibit RXR responsiveness only in the presence of an RAR ligand, suggesting that RXR-binding activity may be restored by RAR ligands. To test this hypothesis, we made use of the observation that 9-cis RA binds with high affinity to both RAR

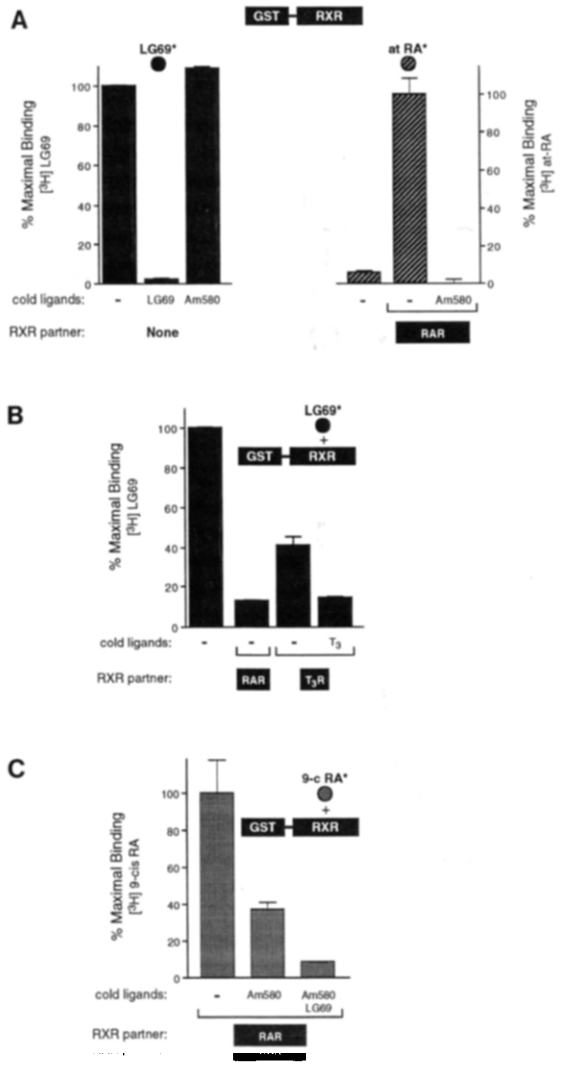

Figure 4. The Ligand-Binding Activity of RXR Is Altered by T_3R and RAR

(A) Binding of LG69 and all-*trans* RA to RXR and RAR. In the experiment shown on the left, purified GST–hRXRα was incubated with 50 nM [^{3}H]LG69 (56 Ci/mmol) and the optimized RAR response element 5′-GCAAAAGGTCAAAAAGAGGTCATGC-3′ (Kurokawa et al., 1993) alone or with 2 μM LG69 and 2 μM Am580. In the experiment shown on the right, purified GST–hRXRα and the RAR response element were incubated with 25 nM [^{3}H]all-*trans* RA (49 Ci/mmol) without or with 500 ng of hRARα. The amount of specifically bound [^{3}H] was then determined as described in Experimental Procedures.

(B) Binding of LG69 to RXR is reduced in RAR–RXR and T_3R–RXR heterodimers. Purified GST–hRXRα and 50 nM [^{3}H]LG69 (56 Ci/mmol) were incubated alone or with 500 ng of hRARα or chicken T_3Rα1 and the optimized RAR response element or the optimized T_3R response element 5′-GCAAAAGGTCAAATAAGGTCACGT-3′ (Kurokawa et al., 1993), respectively. Where indicated, unlabeled T_3 was added to a concentration of 1 μM. Specifically bound [^{3}H]LG69 was determined.

(C) Competition of [^{3}H]9-cis RA bound to RAR–RXR heterodimers requires RAR and RXR ligands. Reactions were performed as in (A) using both GST–hRXRα and hRARα with 50 nM [^{3}H]9-cis RA (29 Ci/mmol). Specifically bound [^{3}H]9-cis RA was determined in the absence or presence of 2 μM LG69, 2 μM Am580, or both. In all experiments, maximal binding was in the range of 200–300 fmol of ^{3}H-labeled ligand.

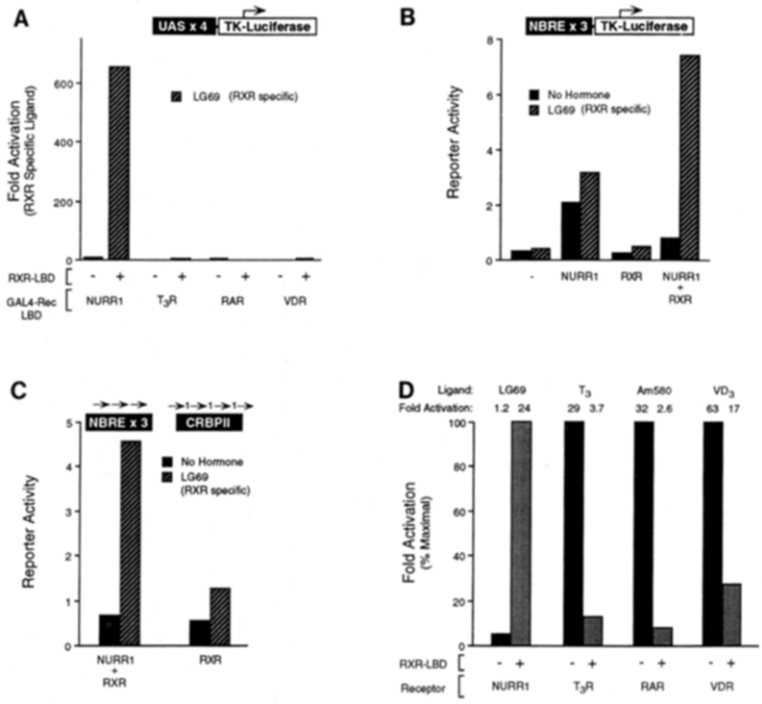

Figure 5. A Novel Nurr1–RXR Complex Provides a Signaling Pathway for 9-cis RA

CV-1 cells were transfected with TK–LUC reporters (300 ng/10^5 cells), CMX–β-gal (500 ng/10^5 cells), and the indicated CMX–receptor construct (20 ng/10^5 cells) with or without CMX–RXR LBD (100 ng/10^5 cells). The following receptor, reporter, and ligand combinations were used: Nurr1, NBRE × 3, 100 nM LG69; $hT_3R\beta$, MLV × 2, 100 nM T_3; hRARα, DR5 × 2, 100 nM Am580; hVDR, SPP1 × 3, 100 nM VD_3. Normalized LUC activity was determined and plotted as percent of maximal fold activation where 100% is defined as the fold activation by Nurr1 and RXR LBD or by T_3R, RAR, and VDR in the absence of RXR LBD. The actual fold activation values are shown above each bar.

(A) Transient transfection analysis of GAL–receptor LBD chimeras in the presence of the RXR LBD. CV-1 cells were transfected with UAS_G × 4 TK–LUC (300 ng/10^5 cells), CMX–β-gal (500 ng/10^5 cells), and the indicated CMX–GAL–receptor LBD construct (100 ng/10^5 cells) with or without CMX–RXR LBD (100 ng/10^5 cells). Following transfection, cells were treated without or with ligand 100 nM LG69. Normalized LUC activity was determined and plotted as fold activation relative to untreated cells.

(B) Transient transfection analysis of full-length Nurr1 and RXR. CV-1 cells were transfected with NBRE × 3 TK–LUC (300 ng/10^5 cells), CMX–β-gal (500 ng/10^5 cells), alone or with CMX–Nurr1 (100 ng/10^5 cells) and CMX–hRXRα (100 ng/10^5 cells) as indicated. Following transfection, cells were treated with or without 100 nM LG69. Normalized LUC activity was determined and plotted as reporter activity.

(C) Comparison of Nurr1–RXR with RXR. Cells were transfected as in (B) but with a 5-fold lower amount of CMX–hRXRα (20 ng/10^5 cells). CRBPII TK–LUC (300 ng/10^5 cells) was used as indicated.

(D) RXR LBD activates through Nurr1 but inhibits activation of other receptors.

and RXR (Allegretto et al., 1993; Allenby et al., 1993). Thus, GST–RXR and GST–RAR heterodimers were allowed to form in the presence of [^{3}H]9-cis RA. Although Am580 fully competed with [^{3}H]all-*trans* RA for binding to GST–RXR and GST–RAR heterodimers (Figure 4A, right), Am580 resulted in only a partial decrease in [^{3}H]9-cis RA binding (Figure 4C). Nearly complete competition was observed by addition of both Am580 and the RXR-specific ligand LG69 (Figure 4C), suggesting that RXR can bind ligand provided that the RAR LBD is occupied. These findings are consistent with the restoration of RXR responsiveness in RAR-occupied heterodimers (see Figure 2B, right).

Identification of a Novel RXR-Permissive Heterodimer

Since RXR served as a silent partner in the T_3R and RAR pathways, we wondered whether RXR could serve as an active component in other complexes. To search for such complexes, we fused the LBD of a number of nuclear receptors to the GAL4 DBD and asked whether the RXR LBD could confer LG69 responsiveness upon these GAL–LBD chimeras. As expected, LG69 responsiveness was not seen when the RXR LBD was expressed alone (data not shown) or with GAL–T_3R and GAL–RAR (Figure 5A). Similarly, LG69 inducibility was not observed with chimeras containing the LBDs of VDR (Figure 5A) or several other members of the nuclear receptor superfamily (data not shown). Unexpectedly, strong responsiveness to LG69 was observed when the RXR LBD was coexpressed with a GAL–Nurr1 chimera (Figure 5A). These results suggest that the LBDs of Nurr1 and RXR form a complex that is responsive to RXR agonists.

Nurr1, the β isoform of NGFI-B, is reported to be a constitutively active orphan receptor that binds as a high affinity monomer to an AAAGGTCA core site, the NGFI-B response element (NBRE) (Law et al., 1992; Wilson et al., 1992, 1993; Scearce et al., 1993). This prompted us to ask whether full-length Nurr1 and RXR could interact productively on the NBRE. Consistent with published results (Scearce et al., 1993), Nurr1 alone constitutively activates the NBRE reporter (Figure 5B) and is not responsive to LG69 (Figure 5B). RXR, which does not bind to the NBRE (data not shown), did not activate this reporter. However, when Nurr1 and RXR are coexpressed, the constitutive activity of Nurr1 is suppressed, and the complex becomes strongly responsive to LG69 (Figure 5B). LG69 and 9-cis RA responsiveness was also observed with the β and γ isoforms of RXR and when NGFI-B was used in place of Nurr1 (data not shown). The ability of Nurr1–RXR to transduce RXR signals led us to compare the activity of this complex with that of RXR on an established RXR response element (CRBPII) (Mangelsdorf et al., 1991). Using suboptimal amounts of an RXR expression vector, we compared the CRBPII reporter with a three-copy NBRE reporter. Since RXR was limiting in this assay, we observed only minimal activation of the CRBPII reporter (Figure 5C). In contrast, Nurr1–RXR displayed a potent re-

sponse to LG69, despite the fact that the NBRE reporter contains one fewer core-binding site than CRBPII (Figure 5C). Thus, Nurr1–RXR can efficiently transduce RXR signals supporting the physiologic relevance of this complex. The Nurr1–RXR complex establishes a novel signaling pathway for 9-cis RA as well as a novel role of RXR in hormonal signaling.

Nurr1 Does Not Require the RXR DBD for Coupling

The Nurr1–RXR complex is unique in several ways. First, the Nurr1 DBD recognizes its response element in the absence of RXR (Wilson et al., 1992, 1993; Scearce et al., 1993). Second, the monovalent NBRE serves as a response element for a multimeric Nurr1–RXR complex (Figure 5B). These observations raise the possibility that RXR associates with NBRE-bound Nurr1 in the absence of RXR-specific DNA contacts. Such behavior would be in sharp contrast with T_3R, RAR, and VDR, which rely on RXR-specific contacts to recognize HREs. Indeed, RXR mutants lacking the DBD associate with wild-type RAR; however, these complexes do not bind DNA or activate transcription (Minucci et al., 1994). This prompted us to ask whether the RXR LBD was sufficient for activation through the Nurr1 pathway. Indeed, as shown in Figure 5D, the RXR LBD confers strong LG69 responsiveness upon coexpressed Nurr1. In contrast, the RXR LBD acts as a dominant negative inhibitor of wild-type T_3R, RAR, and VDR because these complexes require two DBDs to bind DNA (Figure 5D). These findings indicate that the RXR DBD is not required for ligand-dependent activation of Nurr1–RXR, a property that further distinguishes this complex from previously described RXR-containing complexes. Although the monovalent NBRE serves as an HRE for Nurr1–RXR heterodimers, it remains possible that bivalent HREs may also exist for this complex (T. Perlmann, personal communication).

Discussion

The LBD of nuclear hormone receptors is a complex multifunctional unit containing subdomains for dimerization, transcriptional suppression, and hormone-induced transactivation (Forman and Samuels, 1990). The dimerization domain includes a series of heptad repeats flanked by sequences required for ligand binding (Forman and Samuels, 1990). Thus, the dimerization domain is embedded within the larger LBD. This structural arrangement raises the possibility that dimerization may serve as an allosteric modulator of ligand binding and transactivation. We have addressed this issue and established the following points.

Dimerization Modulates Ligand Binding and Transactivation

First, dimerization within the LBD is utilized to confer transcriptional suppression upon a heterodimeric complex. This is exemplified by unliganded T_3R and RAR, which confer transcriptional suppression upon RXR. Reciprocally, we have shown that RXR can suppress constitutive activation by Nurr1.

Second, the intrinsic ligand binding capacity of the LBD can be modulated by dimerization. This is highlighted by the ability of unliganded RAR to abrogate the ligand-binding activity of RXR (Figure 4; Kurokawa et al., 1994). We also find that T_3R suppresses RXR ligand binding but requires T_3 for the complete effect. Thus, RXR serves as a silent partner in the T_3R and RAR pathways. However, not all heterodimeric interactions restrict ligand responsiveness. We show that RXR actively confers ligand responsiveness upon the novel Nurr1–RXR complex, and we have previously shown that the Drosophila ecdysone receptor (EcR) acquires ligand-binding activity after heterodimerization with *ultraspiracle* (Drosophila homolog of RXR) (Yao et al., 1993). Thus, differential interactions among receptor LBDs can either restrict, redirect, or lead to an acquisition of ligand-binding phenotypes.

Allosteric Control: A Structural Model

We propose the following structural model (Figure 6; Forman and Samuels, 1990) to account for our findings. In this model the RXR monomer (or homodimer) is capable of binding ligand with high affinity. When RXR interacts with one of its nonpermissive partners (T_3R or RAR), its ability to bind ligand is diminished. On the other hand, dimerization of *ultraspiracle*/RXR with EcR promotes high affinity binding of ecdysone to EcR. We hypothesize that these effects are a direct consequence of the localization of a major dimerization interface within the LBD (Figures 1A and 6). Our model predicts that this structural arrangement serves functionally to link dimerization and ligand-binding activities. This provides a mechanism for dimerization to exert allosteric control over the ligand response.

In addition to dimerization, ligand binding may result in a second allosteric modification of the partner receptor. Thus, binding of ligand to RAR restores the RXR response; in the case of T_3R, the addition of T_3 further suppresses the response (Figure 4). This has the consequence of making the RAR complex uniquely responsive to retinoids while the T_3R complex is only responsive to T_3. It is already known that upon ligand binding, the cognate receptor undergoes a conformation change (Toney et al., 1993). Our data lead to the prediction that ligand-induced conformation changes in the LBD of one heterodimer partner will be propagated through the dimerization interface onto the LBD of the partner. This model can explain how the dimerization partner and its specific ligand exert allosteric control over the RXR ligand response. This same model can account for the ability of ligand to either promote EcR–*ultraspiracle* (Yao et al., 1993) or destabilize VDR–RXR (MacDonald et al., 1993; Cheskis and Freedman, 1994) and T_3R–T_3R (Andersson et al., 1992; Ribeiro et al., 1992; Yen et al., 1992) dimers.

Physiologic Implications

The restriction of RXR activity within certain heterodimers indicates that 9-cis RA responsiveness is not an obligatory consequence of heterodimerization with RXR. This allows RXR to be both a receptor and heterodimerization partner without requiring all target genes to be 9-cis RA responsive. This explains the paradox as to how RXR serves as a common subunit for receptors that display independent

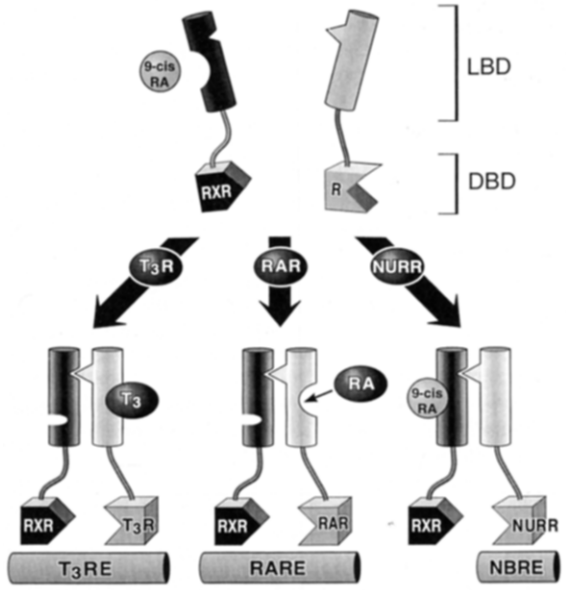

Figure 6. The Allosteric Control Model of Ligand Responsiveness

RXR (dark stippling) and its partner receptor T_3R, RAR, or Nurr1 (R; light stippling) initially exist as monomers in solution. The RXR monomer is capable of binding ligand. Receptor–RXR heterodimers then form, driven by the dimerization interface that is embedded within the LBD. Subsequent to dimerization, binding of 9-cis RA to RXR is modestly reduced by T_3R and dramatically reduced by RAR. Addition of T_3 results in a further reduction in 9-cis RA binding, while certain RAs such as Am580 may restore 9-cis RA binding to RAR–RXR. The Nurr1–RXR heterodimer maintains the ability to bind 9-cis RA. Our structural model relies on the observation that a major dimerization interface is embedded within the larger LBD. We propose that upon dimerization, the structure of the RXR LBD/dimerization domain is altered. Each RXR partner gives rise to unique conformation changes that either maintain or abrogate RXR ligand-binding activity. Binding of ligand by the partner receptor induces a conformation change that can be propagated through the dimerization interface onto the LBD of RXR. This model can explain how the dimerization partner and its specific ligand exert allosteric control over the RXR ligand response.

physiologic effects (e.g., T_3R, RAR, and VDR). We have not explored the mechanisms by which RXR responsiveness is abolished in VDR–RXR heterodimers. This may be related to the observation that 9-cis RA can bind to and destabilize VDR–RXR heterodimers (MacDonald et al., 1993; Cheskis and Freedman, 1994).

In contrast, the ability of RXR to transduce signals when complexed with NGFI-B or Nurr1 suggests an alternative pathway for 9-cis RA signaling. This pathway may regulate the physiology of cells that coexpress RXR and NGFI-B/Nurr1. RXR is found throughout the body with one or more isoforms expressed in virtually all cells and tissues (Mangelsdorf et al., 1992; Titcomb et al., 1994). NGFI-B is expressed in a variety of unstimulated tissues, including the brain, heart, ovary, muscle, and testis (Ryseck et al., 1989; Law et al., 1992), while Nurr1 expression is restricted to the brain (Law et al., 1992; Scearce et al., 1993). Moreover, the expression of both proteins is induced by a number of physiological stimuli (Davis and Lau, 1994), including membrane depolarization, T cell apoptosis (Liu et al., 1994; Woronicz et al., 1994), and liver regeneration (Scearce et al., 1993). We suggest that RXR may contribute to the regulation of these events by functioning as a hormone-dependent cofactor.

The ability of RXR to function as a DNA-dependent protein in one context and a cofactor in another is unique to enhancer-binding proteins. However, this phenomenon has precedence in the TATA-binding protein that participates as a cofactor in RNA polymerase I and III transcription complexes but binds DNA in the RNA polymerase II complex (Pugh and Tjian, 1991; Comai et al., 1992; Cormack and Struhl, 1992; Meyers and Sharp, 1993; Goodrich and Tjian, 1994; Struhl, 1994). An important general conclusion is that heterodimerization is a remarkably effective means to generate diversity of hormone signaling. A more specific conclusion, no less important, is the central role of RXR in this process.

Experimental Procedures

Cell Culture and Transfection

CV-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% resin–charcoal-stripped (Samuels et al., 1979) fetal bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate (DMEM–FBS) at 37°C in 5% CO_2. Cells were plated to 50%–80% confluence 1 day prior to transfection, using phenol–red free DMEM–FBS. Cells were transfected by lipofection using N-{2-(2,3)-dioleoyloxy)propyl-N,N,N-trimethyl ammonium methyl sulfate} (DOTAP) according to the instructions of the manufacturer (Boehringer Mannheim). After 2 hr, the liposomes were removed and cells treated for 40 hr with phenol–red free DMEM–FBS alone or with the following ligands: 100–300 nM T_3 (L-triiodothyronine), 100 nM LG69 (4-{1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-napthyl)-1-ethenyl }benzoic acid), 50–100 nM Am580 (4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthamido)benzoic acid), or 100 nM VD_3 (1α,25-dihydroxyvitamin D3). Cells were harvested and assayed for luciferase (LUC) and β-galactosidase (β-gal) activity. All points were performed in triplicate in each experiment and varied by less than 10%. Each experiment was repeated three or more times with similar results.

Expression and Reporter Constructs

For LUC assays, response elements with HindIII overhangs were cloned into the HindIII site of the thymidine kinase (TK)–LUC reporter that contains the herpes virus TK promoter (−105/+51). Response elements with the underlined consensus hexanucleotide sequence were as follows: UAS_G × 4 (5′-CGACGGAGTACTGTCCTCCGAGCT; four copies); IR0 = TREp (5′-TCAGGTCA TGACCTGAG; one and two copies); DR4 × 2 (5′-AAAGGTCACGAAAGGTCACCATCCCGGGAAAAGGTCACGAAAGGTCACC); DR5 (5′-CAGGTCACCAGGAGGTCAGAG); DR5 x 2 (5′-AAAGGTCACCGAAAGGTCACCATCCCGGGAAAAGGTCACCGAAAGGTCACC); ER8 (5′-TGACCTTTCTCTCCAGGTCA); NBRE × 3 (5′-GAGTTTTAAAAGGTCATGCTCAATTTTC; three copies); CRBPII (5′-GTCACAGGTCACAGGTCACAGGTCACAGTTCA); MLV-DR4 × 2 (5′-CAGGGTCATTTCAGGTCCTTG; two copies); SPP1/osteopontin–DR3 × 3 (5′-AAGGTTCACGAGGTTCACGT; three copies). All mammalian expression vectors were derived from pCMX (Umesono et al., 1991), which contains the CMV promoter/enhancer followed by a bacteriophage T7 promoter for transcription in vitro. pCMX expression vectors for $T_3Rβ$, human RARα (hRARα), (Umesono et al., 1991), and human RXRα (hRXRα) (Yao et al., 1993) were previously described. CMX–Nurr1 (provided by T. Perlmann), an expression vector for full-length mouse Nurr1, was cloned by inserting the BglII–XhoI fragment from pBS34-1 (excised from λZAP34) (Law et al., 1992) into pCMX. The VP16–RXR fusion contains the 78 amino acid transactivation domain of herpes VP16 from pVP16C1 (Novagen) fused N-terminal to full-length hRXRα. GAL4 fusions were made by fusing the following receptor LBDs to the C-terminal end of the yeast GAL4 DBD (amino acids 1–147) from pSG424 (Sadowski and Ptashne, 1989): hRXRα LBD (Glu-203–Thr-462); mouse Nurr1 (Cys-318–Phe-598); human $T_3Rβ$ ($hT_3Rβ$) (Leu-

173–Asp-456); hRARα (Glu-156–Pro-462); and human VDR (hVDR) (Glu-92–Ser-427). The LBD expression constructs contain the SV40 tag nuclear localization signal (APKKKRKVG) fused upstream of the hT$_3$Rβ LBD (Leu-173–Asp-456), the hRARα LBD (Glu-156–Pro-462), or the hRXRα LBD (Glu-203–Thr-462). CMX–β-gal contains the Escherichia coli β-gal coding sequences derived from pCH110 (Pharmacia) cloned into pCMX.

Ligand Binding Assays

Bacterially expressed proteins were used for ligand binding assays. GST–hRXRα (Mangelsdorf et al., 1991), chicken T$_3$Rα1 (Forman et al., 1992), and hRARα (Forman et al., 1992) were expressed and purified to near homogeneity as previously described. GST–RXR (150 ng) or a GST control (150 ng) were incubated with or without approximately 500 ng of T$_3$R or RAR in the presence of 50 nM ^{3}H-labeled ligands (LG69, 56 Ci/mmol; all-*trans* RA, 49 Ci/mmol; 9-cis RA, 29 Ci/mmol), 3 ng/μl poly(dI·dC), 50 fmol/μl of the indicated oligonucleotide, and 10 μl of 50% (v/v) epoxy-linked glutathione–Sepharose (Sigma) in ligand-binding buffer (25 mM Tris [pH 7.8], 0.5% CHAPS, 100 mM KCl, 8% glycerol, 1 mM DTT). Where indicated, unlabeled ligands were added as follows: LG69, 2 μM; Am580, 2 μM; T$_3$, 1 μM. The reaction was mixed for 30 min at 25°C and then chilled to 4°C for 10 min. The glutathione–Sepharose beads were washed three times in ligand-binding buffer, and the amount of [^{3}H] bound was determined in a liquid scintillation counter. Background binding was determined with the GST control and represented 3%–5% of the total binding seen with GST–RXR.

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