LXR, a nuclear receptor that defines a distinct retinoid response pathway

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We have identified a new retinoid response pathway through which 9-cis retinoic acid (9cRA) activates transcription in the presence of LXRo, a member of the nuclear receptor superfamily. LXRo shows a specific pattern of expression in visceral organs, thereby restricting the response to certain tissues. Retinoid trans-activation occurs selectively on a distinct response element termed an LXRE. Significantly, neither RXR homodimers nor RXR/RAR heterodimers are able to substitute for LXRo in mediating this retinoid response. We provide evidence that the retinoid response on the LXRE is the result of a unique interaction between LXRo and endogenous RXR, which, unlike in the RXR/RAR heterodimer, makes RXR competent to respond to retinoids. Thus, the interaction with LXRo shifts RXR from its role described previously as a silent, DNA-binding partner to an active ligand-binding subunit in mediating retinoid responses through target genes defined by LXREs.

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al. 1994]. These findings suggest that for the RXR homodimer to be active [i.e., for RXR to be able to function as a 9cRA receptor in vivo], the ratio of RXR to RAR in a cell must be very high. This may explain why certain cells that endogenously express RXR and RAR (even at low levels) yield significant retinoid responses with DR5-containing reporter genes but do not yield any response with DR1-containing reporter genes unless RXRs are overexpressed in these cells [Mangelsdorf et al. 1991]. Given the ubiquitous expression pattern of RARs and RXRs, one question is whether 9cRA activation of RXR through DR1 elements is the only pathway that exists for mediating a 9cRA–RXR response.

In this paper we report the identification of an orphan member of the nuclear receptor superfamily, named LXRs. In the presence of 9cRA, LXRs is required for trans-activation through a distinct retinoid response element. Our data indicate that the LXRs response to retinoid is attributable to its unique interaction with endogenous RXR in cells. This interaction permits RXR to work as an active, ligand-binding heterodimeric partner. These results demonstrate the ability of LXRs to function as a tissue-specific mediator of a novel 9cRA response pathway.

Results
Molecular cloning of human LXRs

Sequential low-stringency screening of a human liver Agt11 cDNA library with a cDNA probe encoding the human RARα DNA-binding domain led to the isolation of several potential nuclear receptor clones. Preliminary DNA sequencing analysis revealed that a subset of these clones encoded a single novel orphan nuclear receptor. Based on its initial isolation from the liver and its liver-rich expression pattern [see below], this orphan receptor was referred to as LXRs. The longest LXRs cDNA was subcloned [see Materials and methods] and completely sequenced (Fig. 1). Eighteen base pairs downstream from the in-frame termination codon at nucleotide 127 is the predicted translation start codon for LXRs at nucleotide 148. Translation from this ATG predicts an LXRs protein of 447 amino acids (M, 49,000) that we have confirmed by in vitro transcription/translation [data not shown] of the LXRs cDNA depicted in Figure 1. This clone also contains 188 bp of 3'-untranslated region (UTR) that includes a canonical polyadenylation signal and a tract of 33 adenosines of the presumptive poly(A) tail. From these results we conclude that this cDNA encodes the full-length LXRs protein.

A comparison of human LXRs with a number of other known receptors is depicted in Figure 2. Human [h]LXRs shares closest similarity with two recently reported orphan receptor sequences, rat RLD-1 [Apfel et al. 1994] and human NER or UR [Shinar et al. 1994; Song et al. 1994]. Although the functional relationship of these proteins to LXRs is not yet known, one of these receptors, RLD-1, is 92% identical, suggesting that it is the rat homolog of hLXRs. These findings when combined with genomic Southern analysis and evaluation of other LXR-related cDNAs [data not shown] indicate that LXR/RLD-1 constitutes a gene family of at least two members. Interestingly, hLXRs also shares close identity with the Drosophila ecysdrome receptor (dEcR), having a 71% similar DNA-binding domain and 39% similar putative ligand-binding domain. The degree of sequence identity of hLXRs with the human receptors, however, is much lower, but overall it is greatest with the human vitamin D receptor [hVDR]. Of the nonsteroid subclass of receptors with known ligands, hLXRs has the lowest similarity to hRXRs (52% in the DNA-binding domain; 22% in the ligand-binding domain).

LXR expression pattern

Northern blot analyses were performed to determine the tissue distribution and developmental pattern of LXRs gene expression. Poly(A)+ RNA isolated from a variety of adult rat tissues [Fig. 3a] or from staged whole mouse embryos [Fig. 3b] was size-fractionated, transferred to nylon filters, and hybridized with the hLXRs cDNA. The distribution of LXRs mRNA reveals an expression pattern similar to that of RXRs [Mangelsdorf et al. 1990], with strong expression in metabolic organs such as liver, kidney, and intestine. The 1.9-kb LXRs mRNA is also notably present in spleen and to a lesser extent in the adrenals. Analysis of expression during mouse development indicates that low levels of LXRs are detected at embryonic day 13.5 and continue to increase in abundance through parturition.

LXRs trans-activation by retinoids

To identify a potential ligand for the orphan receptor LXRs, we employed a cell-based cotransfection screening assay similar to that used to successfully identify the RXR ligand [Mangelsdorf et al. 1990; Heyman et al. 1992]. Initially, an LXRs-responsive gene was unknown; therefore our preliminary experiments were designed to first identify an LXRs-specific DNA-binding sequence that could serve as an LXRs-responsive element (referred to as an LXRE). During the course of these experiments, we discovered a specific, high-affinity DNA-binding site for LXRs in the promoter sequences of ΔMTV, a promoter derived from the mouse mammary tumor virus LTR [discussed below; see Fig. 6, below]. This discovery suggested that the ΔMTV LXRE sequence could be transferred to a heterologous promoter, such as thymidine kinase (TK), and used with a luciferase (LUC) reporter gene for screening potential LXRs ligands. Thus, an assay system was established by transfecting CV-1 cells with an expression plasmid harboring the cDNA for LXRs and the LUC reporter plasmid TK–LXRE3–LUC [Fig. 4a]. In a comprehensive screen of several classes of compounds, we found that LXRs activation is only induced by the presence of certain retinoid ligands. Remarkably, LXRs is strongly activated by both 9cRA and methoprene acid [MA], which we have shown previously to be an RXR-specific ligand [Harmon et al. 1995]. LXRs and retinoid are both required to confer trans-activation
of the LUC reporter gene containing the LXRE (cf. "Control" with "LXRα" in Fig. 4a). Because these results are similar to the activation specificity of RXRα, a side-by-side comparison of dose responses to 9cRA was performed with LXRα and RXRα on their respective response elements. As shown in Figure 4b, both LXRα and RXRα show nearly identical, concentration-dependent activation profiles in response to 9cRA. Similar dose responses [not shown] were observed with several other RXR-specific ligands, including LG69 (Boehm et al. 1994). These results suggested the existence of a distinct pathway of gene regulation mediated by LXRα and 9cRA.

Three distinct retinoid response pathways
To demonstrate that the LXRα retinoid response is distinguishable from the pathways described previously by the RXR and RAR receptor systems, cotransfection experiments were performed to directly compare the three receptor systems by using receptor-specific ligands and response elements (Fig. 4c–e). For these experiments we used the two retinoid ligands MA and TrNPB, which have been shown previously to be uniquely specific for RXRα and RARα, respectively (Mangelsdorf et al. 1990; Kurokawa et al. 1994; Harmon et al. 1995). Expression experiments were performed to directly compare the three receptor systems, cotransfection experiments were performed to directly compare the three receptor systems by using receptor-specific ligands and response elements (Fig. 4c–e). For these experiments we used the two retinoid ligands MA and TrNPB, which have been shown previously to be uniquely specific for RXRα and RARα, respectively (Mangelsdorf et al. 1990; Kurokawa et al. 1994; Harmon et al. 1995).
LXRα forms a functional heterodimer with RXRα

The data presented above suggest two possible explanations to account for the specific activation of LXRα by retinoids. One possibility is that LXRα is functioning in a fashion similar to other receptors. In this scenario, LXRα trans-activation through an LXR might be mediated by an LXR homodimer or an RXR/LXR heterodimer in which LXR binds the ligand. However, based on the RXR-like specificity of the retinoid response, another plausible explanation is that LXRα is not directly bound and activated by these retinoids but instead is an obligatory heterodimeric partner of endogenous RXR and that retinoid binding (and thus trans-activation) requires the presence of RXR. In either case, it is clear that receptor binding and trans-activation through the LXRE is uniquely dependent on the presence of LXRα, because both the RXR homodimer and RXR/RXR heterodimer fail to activate on the LXRE (Fig. 4c).

Figure 3. Northern analysis of LXRα mRNA in adult and embryonic tissue. Ten micrograms of poly[A]+ RNA from adult rat tissues (a) and mouse embryos (b) from gestation day 9.5 to 18.5 was analyzed in each lane as described in Materials and methods. BRL RNA size markers in kb are aligned at left. The arrows at right represent the approximate size of LXRα-specific transcripts.
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Figure 4. Retinoids specifically induce LXRα on an LXRE. (a) LXRα ligand screen. CV-1 cells were cotransfected with either a control plasmid (Control) or an expression plasmid for hLXRα as indicated in combination with the luciferase reporter plasmid TK–LXRE3–LUC and then incubated with various ligands (see below) or an ethanol control (ETOH). (b) LXRα and RXRα dose response to 9-cis retinoic acid. CV-1 cells were cotransfected with expression plasmids for hLXRα (☐) or hRXRα (○) and the reporter plasmids TK–LXRE3–LUC or TK–CRBP1–LUC, respectively, and then incubated with increasing concentrations of 9cRA. (c–e) LXRα, RXRα, and RARα activation by retinoids is response element specific. CV-1 cells were cotransfected with either a control plasmid (☐) or expression plasmids for hLXRα, hRXRα, or hRARα in combination with reporter plasmids TK–LXRE3–LUC (c), TK–CRBP1–LUC (d), or TK–βRE–LUC (e) and then incubated with various retinoid ligands (see below) or an ethanol control. TK–LXRE3–LUC contains three copies of the LXRE defined in this work, TK–CRBP1–LUC contains the RXRE from the rat CRBP1 promoter (Mangelsdorf et al. 1991), TK–βRE–LUC contains the RAR response element from the mouse RARβ2 promoter (Sucov et al. 1990). Luciferase activity is expressed in relative light units (RLU) and represents the mean of triplicate assays (± S.E.M.) normalized to β-gal as an internal standard. For experiments in c–e, we used two different retinoid ligands that have been used previously to distinguish between retinoid receptor systems: 10⁻⁵ M TTNPB, which is RAR-specific (Mangelsdorf et al. 1990), and 10⁻⁴ M MA, which is RXR-specific (Harmon et al. 1995). The concentrations of ligands used in [a] were 10⁻⁴ M MA and 9cRA, 10⁻⁶ M thyroid hormone (T₃), 10⁻⁶ M 1,25-dihydroxyvitamin D₃ (VD), and 1 mM clofibric acid (CA).

To test the feasibility of these two hypotheses, we first examined the possibility of a heterodimeric interaction between LXRα and one of the retinoid receptors. For these studies we employed a mammalian version of the two-hybrid system originally described for the detection of protein–protein interactions in yeast (Fields and Song 1989). In this strategy the ligand-binding domain of one receptor [i.e., LXRα] is fused to the DNA-binding domain of GAL4, creating a hybrid protein [GAL4–LXRα, Fig. 5a] that can bind to specific upstream activation sequences [UAS₈] but that lacks a constitutive trans-activation domain and therefore cannot by itself activate transcription of a LUC reporter [Fig. 5b]. The use of the LXRα ligand-binding domain as the “bait” in these experiments is based on previous studies that have localized a strong dimerization interface in the ligand-binding domain of other nuclear receptors (Kurokawa et al. 1993; Perlmann et al. 1993). To screen for receptors that may interact with LXRα, the candidate receptors were fused to the 78-amino-acid trans-activation domain of VP16. Each VP16–receptor chimera [Fig. 5a] was cotransfected along with GAL4–LXRα into CV-1 cells [Fig. 5b]. In the presence of two receptors capable of dimerization, the VP16 trans-activation domain is brought into functional proximity with the GAL4 DNA-binding sequences, which in turn permits activation of the promoter driving LUC expression. As is clearly demonstrated in Figure 5b, only the VP16 hybrid protein containing RXRα [but not RARα] is able to induce transcription in the presence of GAL4–LXRα. Similar VP16 hybrids of the VDR and TRs are unable to confer trans-activation [T. Perlmann, unpubl.]. These experiments demonstrate a specific inter-
The putative ligand-binding domain of LXRα contains a functional, RXR-specific heterodimerization domain but not a functional retinoid-binding domain. (a) Schematic representation of GAL4 and VP16 hybrid proteins used in these experiments. The amino acid sequences from each parent protein used to construct the hybrid receptors are shown at the top. For details on construction see Materials and methods. (b) Trans-activation of GAL4–LXRα requires the presence of VP16–RXRα but not VP16–RARα in the mammalian two-hybrid system. In negative control experiments (not shown), none of the VP16 receptors alone are able to confer trans-activation. (c) Ligand specificity of the GAL4–receptor chimeras demonstrates that retinoid responsiveness can be conferred through the ligand-binding domains of RXR and RAR but not LXR. In b and c, CV-1 cells were cotransfected as described in Materials and methods with CMX vectors driving expression of the indicated hybrid proteins and the reporter plasmid TK–MH100x4–LUC, which contains four copies of the GAL4 DNA upstream activation sequence [Kang et al. 1993]. In c ligands were added as described in Fig. 4 except 9cRA was added at 10^{-5} M. Luciferase activity is expressed as a function of RLU corrected for transfection efficiency using an internal β-gal standard and represents the mean of triplicate assays (± S.E.M.).

GAL4–receptor hybrids define ligand-binding specificity

To address the possibility that the retinoid response elicited by LXRα is through the direct interaction of ligand with the putative ligand-binding domain of LXRα, we performed ligand activation experiments with the GAL4–receptor hybrids. Previous studies have shown that the ligand-binding domains of nuclear receptors contain ligand-dependent activation domains [Nagpal et al. 1992]. Thus, when a nuclear receptor ligand-binding domain is fused to the DNA-binding domain of GAL4, this hybrid transcription factor alone can confer ligand-dependent trans-activation of a UASG-containing reporter [Nagpal et al. 1993]. Such a heterologous system provides an effective means for directly assayig receptor–ligand interactions even in the presence of endogenous wild-type receptors. As shown in Figure 5c, the chimeric receptors GAL4–RXR and GAL4–RAR confer ligand-inducible trans-activation that accurately mimics the cognate ligand specificity of wild-type receptor proteins: 9cRA activates both RXR and RAR, MA only activates RXR, and TTNPB specifically activates RAR. In contrast, no activation is seen with the GAL4–LXR chimera in the presence of these same ligands. These results indicate that retinoid receptor ligands cannot directly interact with the LXRα ligand-binding domain when evaluated under similar conditions that permit their interaction with RXR and RAR. Furthermore, the specificity of 9cRA and MA for RXR, taken together with the heterodimerization studies, are strong evidence that the LXRα retinoid response requires the presence of RXRα.

LXR/RXR heterodimers bind and activate through the LXRE

The previous experiments suggest the existence of an LXR/RXR heterodimer as the mediator of retinoid sig-
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To test whether this sequence was specifically bound by an LXR/RXR heterodimer, electrophoretic mobility-shift assays were performed using in vitro-synthesized receptor proteins and 32P-labeled LXRE probe [Fig. 6b]. LXRα and RXRα do not bind to the LXRE probe individually [Fig. 6b, lanes 2, 3], but when mixed together, a strong protein–DNA complex is formed [lane 4]. This complex is supershifted in the presence of RXRα-specific antisera [Fig. 6b, lane 5] but not in the presence of a nonspecific antisera [lane 6]. These results are consistent with the conclusion that only the LXR/RXR heterodimer is capable of high-affinity binding to the LXRE. To demonstrate the functionality of the LXR/RXR heterodimer, an experiment was performed by cotransfecting both LXRα and RXRα with the LXRE reporter in the presence of the RXR-specific ligand MA [Fig. 6c]. As was also demonstrated in Figure 4c, the addition of LXRα alone gives a marked ligand response, whereas RXRα alone gives no response. However, when both LXRαs and RXRα are added together, there is a dramatic synergistic increase in both basal and retinoid responsive activities. The LXR/RXR heterodimer must be exquisitely sensitive to retinoid, because under the conditions these experiments were performed [Fig. 6c], even the addition of one-tenth the amount of RXRα (compared with LXRα) expression plasmid greatly increases the LXR-dependent response. From these experiments we conclude that the presence of endogenous RXR in these cells [Titcomb et al. 1994] is responsible for the ability of LXRα when added alone to confer retinoid responsiveness. This conclusion is consistent with the ability of endogenous RXR to also function as a heterodimeric partner for RAR and other nuclear hormone receptors. The important difference in this case is that RXR does not play the role of a silent partner but rather takes on the role of the active, ligand-binding receptor partner. To our knowledge this is the first report of an instance where endogenous RXR is capable of functioning as a potent retinoid receptor on an element where it acts exclusively as a heterodimer.

The LXRE is a novel DR4 retinoid response element

It has been shown previously that the heterodimers formed between RXR and other nuclear receptors can

Figure 6. The LXRE is a novel functional, high-affinity binding site for LXR/RXR heterodimers. (a) Sequence of the LXRE initially identified at positions –123 to –94 from the transcription start site in the ΔMTV promoter (Hollenberg and Evans 1988). Arrows indicate potential response element half-sites. (b) LXRα binds to the LXRE as an RXR heterodimer. Gel mobility-shift assays were performed as outlined in Materials and methods. The 32P-labeled LXRE oligonucleotide was incubated with in vitro-synthesized LXRα and/or RXRα proteins as indicated in the presence or absence of anti-RXRα antibody [αRXXR] or a nonspecific antibody [ns]. The positions of the LXR/RXR heterodimer, antibody supershifted LXR/RXR (arrowhead), and nonspecific [n.s.] complexes are shown. (c) RXRα synergistically increases LXRα responsiveness to retinoids. Expression plasmids for hLXRα, hRXRα, or no receptor (−) were cotransfected into CV-1 cells in combination with the reporter plasmids TK-LUC or TK-LXRE2-LUC as indicated.

Cells were then incubated with ethanol control (−) or 10⁻⁴ M MA (+) and assayed for luciferase activity as described in Fig. 4. TK–LXRE2–LUC contains two copies of the LXRE. In experiments in which LXR and RXR plasmids were cotransfected together, the ratio of the two expression plasmids was 1:0.1 (25 ng of hLXRα to 2.5 ng of hRXRα). In all other experiments where LXR and RXR were added alone, the amount of each receptor expression plasmid was 25 ng.
bind and activate through DNA response elements that are direct repeats of the core sequence AGGTCA spaced by 1–5 nucleotides (designated as DR1, DR2, DR3, DR4, and DR5) (for review, see Giguhre 1994; Kastner et al. 1994b; Mangelsdorf et al. 1994). Inspection of the LXRE sequence in Figure 6a reveals the presence of two degenerate hexad sequences (underscored by arrows) spaced by 4 nucleotides that resemble the canonical direct repeat. The configuration of the LXRE into a putative direct repeat spaced by 4 nucleotides (i.e., DR4) was examined by performing a competition analysis with 10 different mutant LXRE oligonucleotides in which pairs of nucleotides were systematically changed to cytosine (C) or guanine (G) in the region within and flanking the DR4 motif (Fig. 7a). The ability of mutant LXREs to compete with the labeled native LXRE probe was analyzed using the gel mobility-shift assay. Figure 7a shows that mutant oligonucleotides with substitutions within either half-site of the DR4 motif fail to compete with native LXRE for binding to the LXR/RXR heterodimer even at 50-fold molar excess (lanes 3–8, 13–18). In contrast, LXRE oligonucleotides with similar substitution mutations in the spacer and flanking regions are able to compete well for binding to the LXR/RXR heterodimer (Fig. 7a, lanes 1, 2, 9–12, 19–20). Furthermore, the introduction or subtraction of a single nucleotide into the spacer region of the LXRE to create a DR5 or DR3 motif, respectively, resulted in oligonucleotides that fail to competitively...
bind or be activated by the LXR/RXR heterodimer [data not shown].

From the data presented above, it appears that the minimal LXRE is a DR4-type response element. To further investigate this possibility, a series of perfect AG-GTCA direct repeat oligonucleotides spaced by 0-5 nucleotides [DR0, DR1, DR2, DR3, DR4, and DR5] were used to compete with labeled LXRE for binding the LXR/RXR heterodimer. Figure 7b shows that only the DR4 oligonucleotide [lanes 9,10] can compete comparably to the LXRE [lanes 13,14] for binding to LXR/RXR even at a 10-fold molar excess. Because DR4 sequences are known to bind RXR/TR heterodimers and function as potent thyroid hormone response elements, it was of interest to know if LXRE could also activate through these DR4 elements. As shown, LXRE activation is specific only to the LXRE [Fig. 7c, left] and is not functional on a canonical DR4 thyroid hormone response element [Fig. 7c, right]. In independent experiments, we have also demonstrated that the LXR/RXR can bind but is completely inactive on several other DR4 elements that are known to be thyroid hormone responsive as well. Interestingly, the LXRE is reciprocally a very poor thyroid hormone response element [Fig. 7c, left], in spite of the fact that the RXR/TR heterodimer binds to this sequence with high affinity [data not shown]. These experiments suggest that DNA binding alone is insufficient to permit trans-activation. Thus, the LXRE represents a novel DR4 motif that can distinguish between two hormonal pathways.

Discussion

RXRs are unique in their ability to function as both homodimeric receptors and as obligate heterodimeric partners to receptors in multiple hormone response pathways. The central role of RXR in these pathways is summarized in Figure 8. In the retinoic acid response pathway, as a heterodimer, RXR does not bind ligand but rather serves as a cofactor to RAR in preferentially activating target genes containing DR5 response elements. As a homodimer, the ability of RXR to bind ligand and function exclusively as a 9cRA receptor is limited to DR1-containing target genes. Thus far, activation through the RXR homodimer has been shown to be dependent on the artificial overexpression of RXR, because in all cell types tested, the ubiquitous endogenous expression of RAR and RXR leads to the preferential formation of heterodimers that prevent RXR from binding ligand. This apparent lack of target cells in which the expression of RAR is sufficiently lower than RXR to allow formation of RXR homodimers raises the question as to whether RXR can function in vivo as a 9cRA receptor. In spite of these findings, there is now good evidence that retinoid signaling through RXR can occur in vivo and in cells where both RAR and RXR are naturally expressed [Davis et al. 1994]. Taken together, these results suggest the existence of an alternative pathway that allows RXR to function in vivo as a 9cRA receptor. In this paper we have identified the orphan receptor LXR as a tissue-specific cofactor that permits RXR to function as a potent 9cRA receptor with a distinct target gene specificity. The LXR effect is mediated through a unique heterodimeric interaction, which switches the role of RXR from a silent partner to an active, ligand-binding partner in the heterodimer complex. Significantly, this finding establishes a new pathway by which RXR can function as a bona fide receptor, and further, it defines a third retinoid response system with a novel target gene specificity.

Unlike the RXR homodimer, the LXR/RXR heterodimer response to 9cRA in cells can occur in the presence of endogenous RXR and RAR. In the context of the LXR/RXR heterodimer, the mechanism of RXR activation by ligand appears to be similar to that of RAR. Both receptors require a partner for specific DNA binding and ligand activation. The nature of the LXR/RXR heterodimer, however, is unique in several respects. The heterodimerization of LXR and RXR is specific; we can find no evidence of an LXR interaction with other receptors. Furthermore, the LXR/RXR heterodimer binds to a variety of DR4-like elements but thus far is only ligand responsive on the LXRE. This finding indicates that
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DNA binding alone is not sufficient for ligand inducibility. Recent studies have shown that the polarity of the RXR/RAR heterodimer on the DR5 element is such that RXR sits on the 5' half-site and RAR on the 3' half-site (Kurokawa et al. 1993; Perlmann et al. 1993). In this configuration RAR can bind and respond to ligand. However, on a DR1 element, the polarity of the RAR/RXR heterodimer is reversed and although RAR retains its ability to bind ligand, the complex is no longer ligand responsive (Kurokawa et al. 1994). By analogy, these results suggest that the configuration of the LXR/RXR heterodimer on an LXRE may be different than on other response elements. Thus, it will be of interest to determine the DNA-binding polarity of the LXR/RXR heterodimers in future studies. In addition, we are currently in the process of determining the optimal LXRE sequence for activation by retinoids to facilitate the search for potential target genes. The coincident expression pattern of LXRa and RXRα together with their remarkable similarity to other nuclear receptors suggests that they may have ligands and operate in a fashion similar to the known receptors. Thus far, our data support the notion that it is RXR and not LXR that are able to heterodimerize with RAR and bind to DR4 response elements. The finding of a potential family of orphan receptors is the function of the ever increasing complexity of retinoid action, we note that the one commonality of recent studies has been the discovery of alternative ligands.

Materials and methods

Molecular cloning and analysis of LXR cDNAs

The KpnI–SacI restriction fragment (503 bp), including the DNA-binding domain of hRARα was used to screen a Agt11 human liver cDNA library at low stringency as described previously (Mangelsdorf et al. 1990). Positive clones were isolated, subcloned into pgEM vectors (Promega), restriction mapped, and sequenced by the dideoxy method with Sequenase [U.S. Biochemical] using both single-stranded and double-stranded DNA templates. Sequences were aligned and analyzed by the University of Wisconsin Genetics Computer Group programs [Devereux et al. 1984]. Several LXR clones were isolated; the longest, designated AHL1-1, contained a 2.8-kb insert that was subsequently subcloned into the EcoRI site of pBluescript – KS (Stratagene). Because the size of this insert was identical to that of the vector and contained ~1.2 kb of 5’ sequence that appears to be unrelated to the LXR cDNA [see Fig. 1], the first 1151 nucleotides of this clone were excised by EcoRV digestion at two restriction sites, one within the insert and the other in the multiple cloning site of the vector. The resultant DNA was religated and named pXR2ΔRV. The putative cDNA insert of pXR2ΔRV is 1680 bp [see Fig. 1] and contains the entire coding region of hLXRα as well as a potential polyadenylation signal sequence and 33 bases of the poly(A)+ tail.

Plasmids

Constructions of wild-type receptors inserted into the expression vectors RSV and CMX have been detailed elsewhere (Giguère et al. 1986; Mangelsdorf et al. 1990, Umesono et al. 1991). The hLXRα cDNA insert was excised from the plasmid pXR2ARV with KpnI and BamHI and was ligated into the expression vectors RSV and CMX. Chimeric GAL4–receptor expression plasmids (e.g., CMX–GAL4–LXRα) were constructed by first ligating the GAL4 portion of pSG424 [Sadowski and Ptashne 1989] into the HindIII–SacI sites of pCMX (Umesono et al. 1991) to create pCMX–GAL4. This vector contains the CMV promoter fused to the coding sequence for GAL4(1–147), followed by in-frame polylinker cloning sites and translational stop codons. The cDNAs encoding the ligand-binding domain (LBD) of each of the receptors were then ligated into the polylinker to create GAL4–LBD fusions. The following receptor amino acid sequences were used for these constructions, and their corresponding DNA sequences were ligated into pCMX–GAL4: hLXRα 166–447, hRARα 203–462, and hRARα 186–462. Likewise, chimeric VP16–receptor expression plasmids were constructed by fusing the cDNA fragment encoding the 78-amino-acid trans-activation domain of VP16 [Triezenberg et al. 1988] in-frame to the cDNA encoding the desired nuclear receptor. The VP16–receptor fusions were engineered into CMX expression plasmids. Each chimera contains a 6- to 12-amino-acid linker at the VP16–receptor fusion point to facilitate an in-frame junction. Kozak translational start sites (CAC- CATGG) were engineered into the 5’ end of the VP16-receptor chimeras to provide strong initiator methionines. All fusion points and cloning sites were sequenced, and the full-length proteins were in vitro-synthesized to ensure sequence fidelity. Reporter plasmids for these studies were constructed by ligating the appropriate oligonucleotides into the HindIII site of the TK–LUC vector to create TK–LXRE–LUC, TK–MH100x4–LUC (Kang et al. 1993), TK–DR4x2–LUC (Umesono et al. 1991), TK–CRBP II–LUC (Mangelsdorf et al. 1991), and TK–Cer–LUC (Suv cov et al. 1990). The sense strand of the LXRE oligonucleotide used to construct the reporter plasmids was 5′-agcttCCGGGT-
TCCACGGTATATGACTATCTGAT-3'. All constructs were verified by sequencing.

Cell culture and cotransfection assays

CV-1 cells were maintained at 37°C, 5% CO₂ in DMEM containing 5% calf bovine serum (CBS). Transfections were performed in 48-well plates in media containing 5% dextran-charcoal-stripped CBS by the calcium phosphate coprecipitation technique as described previously [Mangelsdorf et al. 1990]. Eight hours after transfections, ligands were delivered to cells at 10³-fold dilutions [0.1% [vol/vol] of solvent in media]. Retinoids (gifts from Marcus Boehm, Ligand Pharmaceuticals, Inc., San Diego, CA) were manipulated under gold light and stored dark in ethanol or methanol under nitrogen gas at −80°C. Cells were harvested 36 hr after addition of ligand and analyzed for Luciferase and β-galactosidase activity using a Dynatech microtiter plate model ML3000 luminometer and a model MR5000 spectrophotometer, respectively. For most experiments, cotransfection of DNA into mammalian cells was accomplished with 50 ng of reporter plasmid, 50 ng of RSV-βgal or CMX-βgal, 25 ng of each receptor expression plasmid, and pGEM carrier to give 375 ng of DNA/well. For controls, the empty CMX vector was used in place of the receptor cDNA. For the GAL4–receptor chimera experiments, 30 ng of receptor and 80 ng of reporter plasmid were used. For GAL4 and VP16 chimera interaction assays, 30 ng of GAL4–LXRα and 15 ng of the VP16–RAR or VP16–RXR were used along with 80 ng of the reporter plasmid. All transfection data points were normalized using an internal β-galactosidase marker [Mangelsdorf et al. 1990] and represent the mean of duplicate or triplicate assays.

Northern analysis

Murine pol[y(A)]⁺ RNA used in these studies was prepared and assayed by Northern analysis as described previously [Mangelsdorf et al. 1992]. Equal amounts (10 µg) of pol[y(A)]⁺ RNA were loaded in each lane and verified by ethidium bromide staining. The DNA hybridization probe used on each blot was the 534-bp EcoRI fragment of the pXR2ARV cDNA insert. This probe includes nucleotides 1147–1680 of the hLXRα cDNA and encodes part of the ligand-binding domain and 3' UTR of the clone. Autoradiography was for 2 days at −70°C with intensifying screens.

Electrophoretic mobility-shift assays

For electrophoretic mobility-shift assay (EMSA) experiments, receptor proteins were generated using a coupled in vitro transcription/translation system according to the manufacture's instructions [Promega]. Sequences of double-stranded oligonucleotides were as shown in the figures and text and were synthesized with HindIII overhangs. Binding reactions were performed in a total volume of 20 µl consisting of 75 mM KCl, 20 mM HEPES at pH 7.4, 2 mM DTT, 7.5% glycerol, 0.1% NP-40, 2 µg of poly[d(I-C)] [Pharmacia], 40 pmoles of a nonspecific single-stranded oligonucleotide [for removal of nonspecific binding in the lysates], and 2 µl of receptor lysates or unprogrammed [control] lysates. Reactions containing lysates and competing oligonucleotides were incubated for 30 min on ice followed by the addition of 40 pmoles of [³²P]-labeled oligonucleotide probe [labeled by end-filling] and further incubated for 20 min at room temperature. Samples were then analyzed on 5% polyacrylamide gels run in 0.5× TBE buffer. For antibody supershifting experiments, 1 µl of anti-hRXRa antisemur [a gift from Jacki Dyck, The Salk Institute, San Diego, CA] or nonspecific antisemur was added to the reactions after addition of the radiolabeled probe and incubated at room temperature for 10 min, followed by gel electrophoresis.

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