A Novel Nuclear Receptor Heterodimerization Pathway Mediated by Orphan Receptors TR2 and TR4*

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A unique heterodimerization pathway involving orphan receptors TR2 and TR4 is demonstrated. TR2 and TR4 preferentially form heterodimers in solution as well as on DNA elements containing a direct repeat-5 (DR5). The in vitro interaction between TR2 and TR4 is demonstrated by the yeast and the mammalian two-hybrid interaction assays, the pull-down assay, and the gel mobility shift assay. The in vivo interaction is demonstrated by following the intracellular localization of fusion receptors tagged with a green fluorescent protein. The dimerization is mediated by the ligand binding domains, and the three leucine residues on helix 10 of TR2 are critical for this interaction. In addition, coexpression of these two receptors exerts a much stronger repressive activity on a DR5-containing reporter than expressing either receptor alone. In the developing testis, TR2 and TR4 are coexpressed in the same testicular cell populations and exhibit a parallel pattern of expression along development. The preferential heterodimerization between TR2 and TR4 and their coexistence in specific germ cell populations suggest a physiological role of TR2/TR4 heterodimers in germ cell development.

Nuclear receptors constitute a super family of transcription factors that regulate gene expression in a wide variety of biological processes such as growth, differentiation, and development. These transcription regulators modulate the transcription efficiency of their target genes by binding to specific DNA sequences in the promoters of these target genes, thereby recruiting corepressors or coactivators to the transcription machinery (1). The most common form of receptor-DNA interaction is the binding of repeated DNA sequences, either in a direct or an inverted orientation, by dimeric receptors. In most cases, nuclear receptors preferentially form heterodimeric pairs with a common partner, one of the retinoid receptor X (RXR) family members (2). It is widely accepted that RXR family provides the common partners for all the nuclear receptors that are able to form heterodimers.

The orphan receptors belong to the super family of nuclear receptors; however, the biological significance of these orphan members has been debated because of the lack of specific ligands for these receptors. Recently, the biological functions of several orphan receptors have been revealed in gene-targeted mice and by linkage analysis. For example, mice deficient in COUP-TFIIl or hepatocyte nuclear factor-4 displayed embryonic lethality (3), and chromosomal deletion of the RORα gene resulted in a staggered phenotype (4). In addition, it has been suggested that the signaling pathways of some orphan receptors can be coupled to certain established biological pathways, such as the orphan receptor COUP-TFIIl in the bone morphogenetic protein-4 (BMP-4) pathway (5).

We have previously isolated and characterized a mouse orphan receptor TR2–11 gene that is expressed most abundantly in the developing germ cells (6). Later, we have identified two isoforms of this receptor, one encoding 589 amino acid residues designated as TR2-11-f (full-length) (designated as TR2 hereon) and the other encoding 256 amino acid residues as a result of early termination, designated as TR2–11-t (truncated form) (7). The TR2 expression is most abundant in the advanced male germ cells, whereas TR2-11-t is only weakly expressed in somatic cells and early germ cells (7). In several reporter systems, such as a reporter controlled by a direct repeat-5 (DR5)-type RA response element (RARE) of the RARα gene (7) and a reporter controlled by a DR4-type hormone response element of the mouse cellular retinoic acid binding protein I (CRABP-I) gene, we have shown that TR2, but not TR2-11-t, strongly represses the activities of these reporters (8). Other studies have also demonstrated a predominantly repressive effect of TR2 in other putative target gene systems, such as the SV40 promoter (9) and the erythropoietine gene promoter (6).

To understand the molecular mechanisms underlying TR2 actions and to shed light on its associate proteins, we have performed a yeast two-hybrid screening experiment using the ligand-binding domain (LBD) of TR2 as the bait. From an adult testis CDNA library, we have identified several positive clones, including the orphan receptor TR4, or TAK1 (10, 11), suggesting an interaction between TR2 and TR4 mediated by the LBD. Interestingly, TR4 receptor has also been shown to function as a repressor for several reporters, including SV40 promoter and RAR/RXR- and TαR-mediated signaling pathways (11, 12). We then ask whether TR2 and TR4 are able to form receptor heterodimers in vitro/in vivo and if these heterodimers are biologically functional.

In this study, we present several lines of evidence supporting the notion that TR2 and TR4 are able to mediate a unique nuclear receptor dimerization pathway. Neither TR2 nor TR4 forms heterodimer with the RXR members. Instead, these two receptors preferentially interact with each other and exert a
synergistic biological activity when both receptors are present. In addition, these two receptors are coexpressed in the same testicular cell populations and exhibit a temporally parallel pattern of expression in developing testis. The implication of this unique nuclear receptor heterodimerization pathway is discussed.

**MATERIALS AND METHODS**

**Construction of Expression Vectors**—For the yeast two-hybrid system, the full-length mouse TR2, TR4, RARα, and RXRα cDNA were each amplified with the polymerase chain reaction (PCR) and cloned into bait and prey vectors, pBD-GAL4 Cam and pAD-GAL4 (Stratagene, La Jolla, CA), at EcoRI and SalI site. To construct the C-terminal deletions and the point mutation, the DEF domain (residue 166–590) of TR2 (13) was first cloned into the pBD-GAL4 Cam vector at EcoRI and Smal sites (construct TR2DEF), and fragments of various C-terminal deletions were generated by PCR, flanked by HindIII and Smal sites, and used to replace the HindIII/Smal fragment of the wild type vector. The TR2DEF construct was also used as the bait to screen the library described as follows. The LLI-537–539/YFP mutant, with the consecutive three leucine residues (537–539) replaced by YFP, was generated by using a PCR-based point mutagenesis protocol (14). For the mammalian two-hybrid system, the same DEF domain, C-terminal 40 amino acid deletion, and LLL/YPP mutant fragments of TR2, as well as a TR4 DEF domain (182–596) (10), were subsequently cloned into the mamalian version of the bait and prey vectors, pM for GAL4 fusion and pVP16 for VP1 fusion (CLONTECH), respectively.

The green fluorescent protein (GFP) fusions were constructed by placing the cDNAs of the full-length TR2, TR4, and the TR2 DEF domain downstream of the GFP, at BglII and SalI sites (for TR2 and TR4) or Smal (for TR2/DEF) sites of the pEGFP-C1 vector (CLONTECH). The glutathione S-transferase (GST) fusion was constructed by inserting the full-length TR2 to the BamHI site of pGEX-2T vector (Amersham Pharmacia Biotech).

The reporter constructs for the mammalian two-hybrid system were made by placing five copies of the GAL4 binding site (5'-CCGAGGAC- CAGTACTCGG3') upstream of the tk-luciferase reporter. All the expression vectors for transfection experiments in COS-1 cells are under the control of a cytomegalovirus promoter. The full-length TR2, TR4, and A/B deletion of TR2 (99–590) were cloned into pSG5 vector at BglII site for *in vitro* transcription/translation reactions.

**Yeast Two-hybrid Screening and Interaction Assay**—The yeast two-hybrid screening (HybriZAP two-hybrid system from Stratagene) was conducted according to the manufacturer’s instructions. Briefly, the C-terminal 40 amino acid deletion and LLL/YPP mutant fragments of TR2, as well as a TR4 DEF domain (182–596) (10), were subsequently cloned into the mammalian version of the bait and prey vectors, pM for GAL4 fusion and pVP16 for VP1 fusion (CLONTECH), respectively.

The reporter constructs for the mammalian two-hybrid system were made by placing five copies of the GAL4 binding site (5'-CCGAGGAGA- CAGTACTCGG3') upstream of the tk-luciferase reporter. All the expression vectors for transfection experiments in COS-1 cells are under the control of a cytomegalovirus promoter. The full-length TR2, TR4, and A/B deletion of TR2 (99–590) were cloned into pSG5 vector at BglII site for *in vitro* transcription/translation reactions.

**Electrophoretic Mobility Shift Assay**—The mobility shift assay was conducted according to an established protocol (18). Briefly, *in vitro* translated proteins were incubated with 1 ng of probe in 20 μl of binding buffer containing 20 mM Hepes, pH 7.4, 50 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol, 1 μg poly(dI-dC), and 5 mg/ml BSA at 4 °C for 60 min. The protein-DNA complex was analyzed by a 5% polyacrylamide gel in 0.5 X TBE buffer (0.045 M Tris borate, 0.001 M EDTA). The probe was prepared by annealing oligonucleotides containing a RARE of the DR5 type derived from the RARα gene (5'-ACCTTANAGGAGCTCCACCG- AAAGTCTACTCGGATATAAGCCT-3') and labeled with [α-35S]dATP using Klenow enzyme. For competition experiments, 10–100 ng of unlabeled RARE oligonucleotides was included in the reactions. To determine whether the receptors bind DNA as monomers, 100 ng of unlabeled oligonucleotidecontaining a half-site of the repeat (5'-CCGAG- AGTACTCGGATATAAGCCT-3') was included in the reaction.

**Cell Separation From Mouse Testes**—Germ cells from mouse testes were collected and separated by using a CelSep apparatus as described previously (7). Mouse Sertoli cells were isolated by unit gravity separation (19). Briefly, adult testes were decapsulated, incubated with enzyme solution I 1.0% collagenase, 0.2% hyaluronidase, 0.03% DNase, 0.03% soybean trypsin inhibitor in F-12/DMEM solution II (0.1% collagenase/dispase, 0.2% hyaluronidase, 0.05% DNase, 0.03% soybean trypsin inhibitor in F-12/DMEM) each for 30 min at 34 °C with gentle shaking. The testicular cells were subjected to centrifugation, resuspended in 2% BSA in F-12/DMEM, and sedimented for 30 min at 34 °C. The sedimented cells, which contained most of the Sertoli cells, were filtered through a 53-μm nylon cloth, and separated from germ cells by continuing sedimentations five to six additional times.

The methods for RNA isolation, RT-PCR, and primers for TR2 and TR4 and actin were described as previously (7). The primers for TR4 were 5'-TTCTCAGAGGTACCGACG-3' and 5'-CAGGGTCGACAGGCT-3'.

**RESULTS**

**Homo- and Heterodimerization of the Orphan Receptors TR2 and TR4** (14) —By screening a mouse testis cDNA library with the LBD of TR2 as the bait, a total of 61 positive clones was isolated. Among these, four individual clones appeared to be derived from the same mRNA species of the mouse TR4 orphan receptor. Sequence comparison between these two receptors revealed a high homology in helix 10 of the putative LBD, which contained the ninth haptad repeat and was known to contribute to the dimer interface (Fig. 1A) (20). Because of the strong interaction between the TR2 bait and TR4 clones, we then examined the potential interactions among TR2, TR4, and RAR and RXR first in the yeast two-hybrid interaction tests.

The “baits” and “preys” used in this system were constructed by placing each full-length TR2, TR4, RXRα, and RXRβ cDNA downstream of the yeast GAL4 DNA-binding domain (GAL4BD) and activation domain (GAL4AD), respectively. Two reporters, UASGAL4-TATAγGAL4-HIS3 and UASGAL4-TATACHTC1- lacZ, could be activated only when a specific interaction occurred between the bait and the prey, as indicated by the growth on histidine-deficient medium and an β-galactosidase (LacZ) activity. Different combinations of the baits and the preys were cotransformed into yeast, which was then plated on a selection medium. A liquid lacZ assay was performed to examine the interaction. As shown in Fig. 1B, the homodimeric interaction of TR2 or TR4 and the heterodimeric interaction of RAR/RXRβ result in 30–50 units of β-galactosidase activities in the protein interaction tests. Interestingly, the interaction between TR2 and TR4 induces a β-galactosidase activity of 75 units, indicating a much stronger interaction between TR2 and TR4. Surprisingly, neither TR2 nor TR4

**Heterodimers of TR2 and TR4 Receptors**

Reductase and LacZ assays were performed as described previously (17). Each experiment was carried out in triplicate cultures, and at least three independent experiments were conducted to obtain the means and standard error of the mean (S.E.). For the expression of GFP fusion proteins, COS-1 cells were grown on a cover glass in a 3-cm dish and incubated with DME containing dextran charcoal-treated serum (DCC medium). Forty-eight hours after transfection, cells were fixed with 4% formaldehyde and visualized by microscopy.
interacts with RAR\(_a\) or RXR\(_a\), the common heterodimer partner, in the presence or absence of retinoic acid. As expected, the negative control (GAL4BD/GAL4AD) induces no LacZ activity and the positive control (p53/SV40) induces a moderate LacZ activity.

To confirm the heterodimerization of TR2/TR4, a GST pull-down assay was performed. The full-length TR2 was fused to a GST expression vector, expressed in \(E.\ coli\), applied to a glutathione-Sepharose column, and subsequently incubated with \(\text{in vitro}\) translated \(35\text{S}\)-labeled TR4. Following an extensive washing procedure, the bound protein was eluted and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1C, TR4 is coeluted with the GST-TR2 fusion, but not the control GST protein, indicating a specific interaction between TR2 and TR4.

To further examine if TR2 and TR4 could heterodimerize on the putative DNA response element, we performed an electrophoretic mobility shift assay using a commonly used DNA element for these receptors, the DR5-type repeat derived from the RAR\(_b\) promoter (7). Radioactive labeled DNA fragments containing the DR5 were incubated with \(\text{in vitro}\) translated receptor proteins, separated by a polyacrylamide gel, and examined by autoradiography. To differentiate the two receptors that migrated at very similar positions when bound to this DNA element, we constructed an A/B domain deletion of TR2 that retained the ability to bind DNA. As shown in Fig. 1D, TR2 (the A/B deletion), as well as TR4, each binds DNA as ho-
modimers (lanes 2 and 3, respectively). As expected, a major band representative of the heterodimers of TR2 and TR4 is observed in the reaction that the DNA fragments are coincubated with both receptors (lane 3). Interestingly, the bands representing either TR2 or TR4 homodimers appear as minor bands, indicating preferential formation of TR2/TR4 heterodimers on the DNA elements. To demonstrate a specific interaction of this heterodimeric receptor pair with the DNA element, a competition experiment was included as shown in lanes 5–7. The retarded band representing TR2/TR4 heterodimers is competed, in a dose-dependent manner, by the addition of the cold DNA fragments (lanes 5–7), but not by DNA fragments containing only a half-site of the repeat (lane 8). Lane 1 shows the reaction containing the reticulocyte lysate and the probe, as a control of nonspecific interaction in this system. These results demonstrate that although both TR2 and TR4 can form homodimers as reported by all the previous studies (8, 21), they preferentially form heterodimers on the putative DNA response element.

Taken together, it is concluded that TR2 and TR4 preferentially heterodimerize with each other, although both are able to form homodimers of their own, in solution as well as on DNA elements. Unlike most nuclear receptors that are able to form heterodimers, TR2 and TR4 do not form heterodimers with RXRs, the common partners in nuclear receptor heterodimerization.

**Helix 10 in Heterodimerization of TR2 and TR4—**Dimerization of nuclear receptors is mediated primarily by their LBD. The crystal structures of RXRa, RARa, and T3Ra reveal the dimer interface formed mainly by the helix 10 of their LBDs (20, 22, 23). To determine the heterodimer interface of TR2, a series of C-terminal deletions and a point mutation of the LBD (the DEF domain) of TR2 were constructed in GAL4-BD fusions (Fig. 2, upper panel). These GAL4-BD fusions of TR2 were tested for their abilities to interact with GAL4AD-TR4 in the yeast by using a liquid LacZ assay. As shown in Fig. 2, TR2 mutations with deletion of 40 and 50 amino acid residues from the C terminus (constructs C∆40 and C∆50, respectively), which are truncated in either the entire helices 11 and 12 (C∆40) or parts of the helix 10 (C∆50), lose the abilities to interact with TR4. In contrast, the 10, 20, and 30 amino acid deletions (constructs C∆10, C∆20, and C∆30, respectively), in which only helix 12 and parts of helix 11 are disrupted, have no effect on the interaction with TR4. Like other receptors (20), the conserved leucine residues within the ninth heptad repeat of the helix 10 are also important for this heterodimeric interaction, as evidenced by the failure of interaction between a TR2 point mutation with the 3 leucine residues changed to YFP (the construct LLL/YPP) and TR4. It is concluded that helix 10 of TR2 is important for the formation of heterodimers.

To determine whether TR2/TR4 interact in mammalian cells, a mammalian version of the two-hybrid interaction test was conducted. The wild type and mutated TR2 as well as TR4 were each fused to the bait and the prey vectors in the same manner as in the yeast system, with the GAL4AD replaced by the VP16AD (24). The upper panel of Fig. 3 shows the reporter, a tk-luciferase reporter containing five copies of the GAL4 binding site and the expression vectors under the control of the SV40 promoter. The reporter and different combinations of GAL4BD and VP16AD fusions were cotransfected, along with a SV40-lacZ as an internal control, into COS-1 cells. The relative luciferase unit (RLU) was calculated by normalizing the luciferase units to the lacZ units. As shown in Fig. 3, the interaction of the TR2 with itself (BD-TR2DEF and VP16-TR2DEF) results in a 2.5 higher reporter activity, whereas the interaction between TR2 and TR4 (BD-TR2DEF and VP16-TR4DEF) results in a 12.5-fold higher reporter activity (Fig. 3), again indicating a stronger interaction between TR2 and TR4 than the homodimers in mammalian cells. As predicted, both of the C-terminal 40-amino acid deletion (BD-TR2C∆40) and the leucine point mutation (BD-TR2LLL/YPP) fail to interact with TR4. A similar result was obtained when TR2 and TR4 were switched between the bait and the prey vectors (BD-TR4 and VP16-TR2) (data not shown). It is concluded that an intact helix 10 is required for efficient TR2/TR4 heterodimerization in both the yeast and the mammalian cells, since all mutations (deletion or point mutation) that disrupt helix 10 of TR2 lose their abilities to heterodimerize with TR4. In addition, the interaction of TR2/TR4 heterodimers is approximately six times stronger than that of either TR2 or TR4 homodimers.

**Synergistic Repression of RA Signaling Pathway by TR2/ TR4 Heterodimers—**Both TR2 and TR4 have been shown to repress RA induction of reporters containing a DR5-type reti-
noic acid response element (RARE) derived from the RARβ promoter through specific DNA binding of homodimeric receptors (7, 21). Since TR2 and TR4 preferentially formed heterodimers when both receptors are present (Fig. 1D), we then examined the effects of TR2/TR4 heterodimers on the RA signaling pathway using this reporter system. The RARE-tk-luciferase reporter was cotransfected with the TR2 vector, the TR4 vector, or the combination of TR2 and TR4, along with a SV40-LacZ internal control, into COS-1 cells. RA was added to a final concentration of 10^{-7} M. The fold of reporter induction by RA was calculated by comparing the RLU in the presence of RA to that in the absence of RA. RA consistently induces this reporter for approximately 100-fold. Consistent with the previous reports, expression of TR2 or TR4 alone represses the induction level in a dose-dependent manner (Fig. 4). Interestingly, the combination of TR2 and TR4 exerts a stronger repression on RA induction of this reporter (with an equal amount of total receptor DNA added in each transfection). Furthermore, this synergism is also dose-dependent, and the repression of RA induction is abolished when the wild-type TR2 expression vector is replaced with the vector carrying the point mutation (TR2-LLL/YPP) that cannot heterodimerize with TR4. From these data, it is concluded that the presence of both TR2 and TR4 exerts a synergistic repression of RA signaling pathway, and this synergism is mediated by heterodimeric TR2/TR4 binding to a specific DNA sequence.

TR2 and TR4 Interact in Vivo—Nuclear receptors can be divided into two categories based upon their subcellular distributions. The steroid hormone receptors are present in the cytosol, and ligand binding induces their nuclear translocation. On the other hand, most other receptors such as T3Rs, RARs, and RXRs are localized in the nuclei. We have determined a specific nuclear localization signal of TR2 within the DNA-binding domain of this receptor and found that truncation of the DNA-binding domain results in predominantly cytosolic localization.2 To determine TR4 localization and to examine the interaction of TR2 and TR4 in vivo, we employed a GFP fusion strategy to tag these receptors. The full-length TR2, TR4, and a truncated TR2 with its A/B/C domain deleted, were each fused downstream to the GFP vector, transfected into COS-1 cells maintained in medium supplemented with dextran-charcoal-treated serum (DCC medium), and visualized by fluorescent microscopy. In these experiments, both TR2 and TR4 exhibit a constitutive nuclear localization pattern (Fig. 5, A and 2 Yu, Z., Lee, C.-H., chinasial, C., and Wei, L.-N. (1998) J. Endocrinol., in press.

Fig. 3. Interaction of TR2/TR4 in mammalian cells. A mammalian version of the two-hybrid system was utilized to examine the interaction between TR2 and TR4. The upper panel shows a luciferase reporter with five copies of GAL4 binding site and all the expression vectors. The GAL4AD was replaced by a VP16 activation domain in this system. Different combinations of GAL4BD and VP16 fusion vectors were cotransfected, along with a SV40-lacZ as an internal control, in COS-1 cells. Forty-eight hours after transfection, cells were harvested, and luciferase and β-galactosidase activities were determined. The RLU was calculated by normalizing the specific luciferase activity to that of the LacZ activity.

Fig. 4. Synergistic repression of an RA signaling pathway by TR2/TR4 heterodimer. A RARE-containing luciferase reporter was cotransfected with TR2, TR4, or a combination of TR4 and TR2 (or TR2-LLL/YPP mutant), along with a SV40-lacZ internal control construct, into COS-1 cells. RA (10^{-7} M) was added 24 h after transfection. The RLUs were determined as described in the legend to Fig. 3, and the fold of induction was determined by comparing the RLU in the presence of RA to that in the absence of RA. The amount of expression vectors used in each combination was indicated.

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B), the GFP fusion of truncated TR2 is predominantly cytosolic (Fig. 5C), whereas the GFP itself is distributed evenly within the cells (Fig. 5E). Interestingly, the truncated TR2 is accumulated in the nuclei in the presence of untagged TR4 (Fig. 5D), indicating trapping of the otherwise cytosolic, truncated TR2 inside the nuclei by TR4. This phenomenon cannot be seen in parallel experiments using either the RAR or RXR expression vectors (data not shown). These results clearly demonstrate that both TR2 and TR4 are localized in the nuclei and that TR2 and TR4 are able to interact in vivo, mediated by the LBD.

**Coexpression of TR2 and TR4 in Developing Testes**—TR2 was most abundant in the adult testis, whereas TR4 was expressed in most tissues examined, including testis (10, 11). To determine whether these two receptors were expressed in the same cell populations of the testis, RT-PCR experiments were conducted to examine the expression patterns of these two receptors in purified testicular cells and in mouse testes at different developmental stages. Total RNAs were isolated from pachytene spermatocytes, round spermatids, Sertoli cells, and testes collected from animals at different ages. RT-PCR experiments were performed by using primer pairs flanking the C-terminal and the N-terminal portions of the TR2 and TR4 cDNAs. The primers for actin were included for an internal control. The products were separated by electrophoresis, transferred to a nylon membrane, and hybridized to specific probes. A typical result following hybridization is shown in A, and a semiquantitative result following phosphorimager quantitation and normalization to the actin control is shown in B. The highest relative value of TR2 and TR4 after normalization was each given an arbitrary value of one.

| B | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| TR2 | 0.75 | 0.50 | 0.25 | 1.00 |
| TR4 | 0.25 | 0.50 | 0.75 | 1.00 |

**DISCUSSION**

This is the first demonstration of heterodimer formation of two different orphan nuclear receptors, which interact both in vitro and in vivo, dimerize on RA response elements, and exert synergistic biological activities without the participation of RXR family members. In studies presented here, we provide several lines of evidence for this unique nuclear receptor heterodimerization pathway. These two receptors preferentially form heterodimers with each other in solution as well as on DNA elements, although they both can also form homodimers of their own. The dimerization is mediated by their LBDs and the three leucine residues on helix 10 of TR2 are important for this interaction. In addition, these two receptors exhibit a
synergistic biological activity as demonstrated by the stronger repression of the reporter activity in the presence of both receptors. In the developing testis, TR2 and TR4 are coexpressed in the same testicular cell populations and exhibit a parallel pattern of expression along development, suggesting a physiological role of TR2/TR4 heterodimers in germ cell development.

Dimerization is essential for most nuclear receptor functions. Steroid hormone receptors and orphan receptors hepatocyte nuclear factor-4 and germ cell nuclear factor form homodimers, whereas RAR, T3R, vitamin D receptor, and orphan receptors LXR and FXR all form heterodimers with the common partner RXR. Exceptions are isomers of COUP-TF, which form both homodimers and heterodimers (with RXR), and ERR, which binds DNA as monomers (2). Surprisingly, neither TR2 nor TR4 is able to interact with RXRs despite a highly homologous helix 10 among these receptors. It is suggested that TR2 and TR4 family members constitute a unique receptor dimerization pathway without the participation of RXR family members. However, it remains to be determined whether TR2/TR4 heterodimers are able to heterodimerize with other receptors. Recent studies have reported that heterodimers can be formed between estrogen receptors α and β, as well as between glucocorticoid receptor and mineralocorticoid receptor, based upon gel mobility shift assays (25, 26). Our studies provide several lines of direct evidence for TR2 and TR4 heterodimer formation both in vitro and in vivo.

The homodimers of TR2 and TR4 bind to the DNA elements that are also the targets of different receptor pairs involving RXR, such as RAR/RXR and T3R/RXR (7, 8, 21). In addition, the binding of TR2 or TR4 to these DNA elements is much stronger than those using the RXR as heterodimeric partners (8, 27), suggesting a competitive role of these orphan receptors in hormonal signaling pathways. Many putative target genes for TR2 and TR4 have been examined, including the cellular retinoic acid-binding protein I gene (8), the erythropoietin gene (6), the RARβ promoter (7), and the SV40 promoter (9, 12). The discovery of TR2/TR4 heterodimeric pathway raises questions as to whether the regulations of these genes by TR2 and TR4 are mediated by homodimers or heterodimers and how the TR2/ TR4 dimeric pathway may modulate hormonal regulation of these genes under a physiological condition.

The coexpression of TR2 and TR4 in particular germ cell populations suggests a physiological role of these heterodimeric receptors in specific spermatogenetic or meiotic events. Since TR4 is highly expressed in most tissues including germ cells, whereas TR2 is specifically elevated only in meiotic germ cell populations, it is possible that the specificity of TR2/TR4 heterodimers is contributed primarily by TR2. Many regulatory events must take place in these cells to control the stability of genetic material and proper cellular differentiation. Vitamin A is essential for these events, since vitamin A depletion results in germ cell arrest at the preleptotene stages and the loss of meiotic cell populations (28). These orphan receptors may modulate vitamin A signaling pathways by employing a separate signaling system, mediated by their unique heterodimers, in the developing germ cells.

Both TR2 and TR4 have been shown to repress promoter activities of several genes in a DNA binding-dependent manner in the presence of regular or dextran charcoal-depleted serum (6, 8, 9, 12). Our recent study has demonstrated that the LBD of TR2 encodes a transferable, repressive activity when it is tethered to DNA elements (29). Furthermore, this active repression does not involve the common corepressor N-CoR (29). This finding suggests a repressive signaling pathway that may have been adopted by TR2 and differs from that utilized by other receptors such as RARs or T3Rs (1). Most orphan receptors are found to be repressive, including TR2 and TR4; however, examples exist that can activate target genes in the absence of potential ligands, such as hepatocyte nuclear factor-4 (2). Moreover, a recent study demonstrates the presence of a tissue- and stage-specific transcriptional coactivator, UTF1 (30). It is highly possible that orphan nuclear receptors could encode activating or repressing activities in vivo, depending upon the nuclear environment such as the status of ligands and specific cofactors in certain cell types or during a particular developmental stage. The repressive activity of TR2 and TR4 in COS-1 cells, as demonstrated in this study, by no means represents the full spectrum of their biological activities in a physiological condition. We are now investigating other coregulators for the TR2/TR4 family.

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