The Orphan Nuclear Receptor TR4 Is a Vitamin A-activated Nuclear Receptor*5°

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Testicular receptors 2 and 4 (TR2/4) constitute a subgroup of orphan nuclear receptors that play important roles in spermatogenesis, lipid and lipoprotein regulation, and the development of the central nervous system. Currently, little is known about the structural features and the ligand regulation of these receptors. Here we report the crystal structure of the ligand-free TR4 ligand binding domain, which reveals an autorepressed conformation. The ligand binding pocket of TR4 is filled by the C-terminal half of helix 10, and the cofactor binding site is occupied by the AF-2 helix, thus preventing ligand-independent activation of the receptor. However, TR4 exhibits constitutive transcriptional activity on multiple promoters, which can be further potentiated by nuclear receptor coactivators. Mutations designed to disrupt cofactor binding, dimerization, or ligand binding substantially reduce the transcriptional activity of this receptor. Importantly, both retinol and retinoic acid are able to promote TR4 to recruit coactivators and to activate a TR4-regulated reporter. These findings demonstrate that TR4 is a ligand-regulated nuclear receptor and suggest that retinoids might have a much wider regulatory role via activation of orphan receptors such as TR4.

Orphan nuclear receptors constitute a group of nuclear receptors (NRs) that lack known functional ligands (1). Recent evidence has shown that orphan nuclear receptors play key roles in many essential physiological functions and can be used to identify therapeutic targets for human diseases (2–6). In contrast to those of classic hormone receptors, however, the functions of many orphan receptors remain unclear because the investigation of their physiological functions is limited by the lack of information about their structural features and their functional ligands.

Testicular receptors form a subgroup of orphan nuclear receptors that consists of two members, TR2 and TR4 (7, 8). TR2 and TR4 are expressed in spermatocytes, erythroid cells, liver, and some regions of the brain and are known to play roles in spermatogenesis, lipid and lipoprotein regulation, and the development of the central nervous system (7, 8). TR4 binds to the direct repeat AGGTCA with a variable number of spacer nucleotides (from 0 to 6) in the promoters of putative target genes (9). TR2 and TR4 homodimerize and heterodimerize with each other but do not heterodimerize with retinoid X receptor (RXR) members. The TR2/TR4 heterodimer binds to direct repeats of DNA elements in the embryonic and fetal globin gene promoter and represses globin gene transcription (10, 11).

The domain organizations of both TR2 and TR4 are similar to those of many other NR family members. Located in the middle of the receptor is a DNA binding domain (DBD), and mapped to the C terminus is the ligand binding domain (LBD) with a ligand-inducible activation function region, AF-2. Located at the N terminus and upstream of the DBD is the N-terminal domain (NTD), including one or two activation function regions (AF-1), which is constitutively active for many NRs. Based on phylogenetic tree analysis (12), TR2 and TR4 belong to the subfamily 2 of nuclear receptors that include RXRs and the chicken ovalbumin upstream promoter-transcription factors (COUP-TFs). Members of the subfamily 2 nuclear receptors share 40–50% sequence identity in their LBDs. Ligand-free structures of RXRα and COUP-TFIIs reveal a common feature of an autorepressed conformation with their helix 10 bending into the ligand binding pocket and with their AF-2 helices occupying the coactivator binding sites. Furthermore, all RXRs and COUP-TFIIs are known to be activated by retinoic acids (13–15).

Although TR2 and TR4 play important physiological roles in humans and animals, the associated signaling pathway and mechanism of action remain elusive because we lack information about their three-dimensional structures and their activation by endogenous ligands. We have determined the crystal structure of the ligand-free TR4 ligand binding domain...
and demonstrated that TR4 is a retinol-activated nuclear receptor. The crystal structure of the TR4 LBD revealed an autoepressed conformation, resembling the ligand-free conformation of RXRα and COUP-TFII. Importantly, coactivator recruitment and transcription activation by TR4 can be enhanced by retinol binding, indicating that TR4 is a ligand-regulated receptor in which retinol activates the receptor by converting its autoepressed LBD into an active conformation. The identification of retinol as an activating ligand for TR4 further establishes the functional connection between retinoid signaling and nuclear receptors beyond the classic retinoic acid receptors.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The human TR4 LBD (residues 367–601, with Lys-539 and Lys-550 mutated to alanine) was expressed as a His$_6$-GST fusion protein from the expression vector pET24a (Novagen). BL21 (DE3) cells were grown to an A$_{660}$ of $-1.0$ and induced with 50 μM isopropyl β-D-thiogalactopyranoside at 18 °C. Six liters of cells were harvested and resuspended in 200 ml of extract buffer (10 mM Tris, pH 7.3, 200 mM NaCl, and 10% glycerol) and followed by $-50$ μg of lysozyme, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), and 100 μM PMSF. Cells were passed through a French press at a pressure of 1,000 pascals, and the lysate was centrifuged at 20,000 × g for 30 min. The supernatant was added over a pre-equilibrated 25-ml glutathione-Sepharose fast flow column (Amersham Biosciences). The column was washed with 200 ml of wash buffer (10 mM Tris, pH 8.0, 1 mM NaCl, 10% glycerol, and 0.1% Triton X-100) followed by 300 ml of buffer A (10 mM Tris, pH 8.0, 100 mM NaCl, and 10% glycerol). The protein was eluted using buffer A supplemented with 4 mM reduced glutathione. The His$_6$-GST-TR4 fusion protein was cleaved overnight with thrombin (0.5 NIH units/mg of fusion protein) at 4 °C. The cleaved TR4 protein was loaded onto a pre-equilibrated 10-ml nickel-chelating Sepharose column (Amersham Biosciences) and eluted at 8% buffer B (500 mM imidazole in 10 mM Tris, pH 8.0, 1 mM NaCl, 10% glycerol). EDTA and DTT were added to 1 mM, and the protein was concentrated for crystallization. A typical yield of the purified TR4 LBD was about 4 mg/liter cells.

**Crystallization and Data Collection**—Crystals of the TR4 LBD were grown at 20 °C in hanging drops containing 3.0 μl of the above protein solution and 1.0 μl of well buffer containing 20% PEG 3350, 0.1 M Bis-Tris, pH 8.0, and 0.2 M ammonium sulfate. Crystals appeared within 3 days and grew to $-300–500$ μm over 1 week. TR4 crystals were soaked in 20% glycerol in the well buffer. All crystals were flash-frozen in liquid nitrogen before data collection. The TR4 crystals formed in the F222 space group with $a = 129.48$ Å, $b = 140.96$ Å, and $c = 184.86$ Å and $α = β = γ = 90^\circ$ (see Table 1). The datasets were collected with a MAR225 CCD detector at the ID line of sector 5 of the Advanced Photon Source at Argonne National Laboratory (Argonne, IL). The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package (16).

**Structure Determination and Refinement**—The CCP4 program PHASER was used for molecular replacement (17), with the COUP-TFII LBD structure (Protein Data Bank (PDB) code: 3CJW) (15) as the search model. The initial model was manually rebuilt and refined using CNS (18) and the CCP4 program REFMAC. The final model included all but 18 residues in the loop region between helix 5 and helix 7. The pocket volume and surface were calculated with the program VOIDOO (19) using program default parameters and a probe with a radius of 1.4 Å. All figures were prepared using PyMOL (20).

**Transient Transfection Assays**—The expression plasmids of human TR4 were constructed by inserting the TR4 cDNA into the mammalian expression vector pCR3.1. The plasmids of PGC1α, SRC1, SRC2, and SRC3 were previously described (15, 21). The luciferase reporter plasmids were constructed by inserting the oxytocin promoter in the pG3-luc (Promega) vector. The nerve growth factor inducible A promoter luciferase reporter plasmid (22) and the phosphoenolpyruvate carboxykinase promoter luciferase reporter plasmid (23) were previously described. All mutant TR4, PGC1α, and SRC-3 plasmids were created using the QuikChange Kit (Strategene).

For cell-based assays, COS-7 cells were maintained in DMEM containing 10% fetal bovine serum (FBS). Cells were transiently transfected in Opti-MEM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Twenty-four-well plates were inoculated with 50,000 cells 24 h prior to transfection. Each well of cells was transfected in Opti-MEM with 150 ng of reporter plasmid and 0.5 ng of Renilla luciferase expression plasmid pRL-CMV (Promega) in all experiments.

For coactivator experiments, cells were transfected with 150 ng of TR4 expression vector and either wild-type or mutant coactivators. For wild-type and mutant TR4 transfections, 150 ng of DNA were used in each experiment. At 24–30 h after transfection, cells were harvested, and the firefly and Renilla luciferase activities were measured.

For the ligand activation assay, 50,000 COS-7 cells were plated in a 24-well plate 24 h before transfection. Cells were transiently transfected with 150 ng of TR4 expression vector and 150 ng of reporter plasmid, and 0.5 ng of phRL-CMV (Promega). At 14 h after transfection, the medium was changed, and retinoid compounds were added. Cells were incubated for another 24 h and harvested for a luciferase assay that used the Dual-Luciferase reporter assay system (Promega). Firefly luciferase values were normalized to Renilla luciferase, which was used as an internal transfection control. All assays were performed in triplicate, and standard deviations were calculated using the triplicate data.

**Ligand Binding Assays**—Ligand binding to TR4 was determined by the ability of the ligands to promote TR4 to recruit coactivator peptides, which was measured by an AlphaScreen assay (PerkinElmer Life Sciences) as described for other NRs (24). The TR4 LBD protein was purified as a His$_6$-GST fusion protein for the assays. The experiments were conducted with $-0.4$ μM receptor LBD and 0.1 μM biotinylated SRC3-3 peptide (KENNALLRYLLD6D) in the presence of 20 μg/ml donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 50 mM CHAPS, and 0.1 mg/ml bovine serum albumin, all adjusted to a pH of 7.4. To determine the
binding affinity of retinoids for the TR4 LBD, retinol (Sigma-Aldrich) and all-trans-retinoic acid (atRA, Enso Life Sciences) were each added to a concentration of 50 μM. EC$_{50}$ values for retinol and atRA were determined from a nonlinear least-squares fit of the data based on an average of three repeated experiments, with standard errors typically less than 10% of the measurements.

RESULTS

Crystal Structure of the TR4 LBD—A series of the human TR4 LBD with variation in the N terminus was screened for expression and crystallization. The final construct of the human TR4 LBD (amino acids 367–615) was purified in the absence of ligand and was crystallized in space group F222 with a dimer in one asymmetric unit (Fig. 1A). The structure was solved at a resolution of 3.0 Å by molecular replacement using the COUP-TFII LBD structure (PDB code: 3CJW) (15) as a search model. The statistics of data collection and model refinement are listed in Table 1.

Fig. 1B shows the monomeric structure of the apo-TR4 LBD, which adopts a three-layered sandwich formed by 10 helices. Two monomeric structures are packed against each other’s N-terminal portion of helix 10 to form a symmetric dimer (Fig. 1A). The overall structure of the TR4 LBD resembles the LBD structures of RXRα and COUP-TFII with its key features described below.

The first key feature of the TR4 LBD is the absence of the N-terminal helix 1. Based on the sequence alignment, we expected that the N terminus of the TR4 LBD in the expression construct could encompass a helix the same as the helix 1 of COUP-TFII and RXRα (supplemental Fig. S1). However, this region of TR4 adopts a long extended loop that partially overlaps the corresponding space occupied by this helix 1 in COUP-TFII (Fig. 1A and supplemental Fig. S2). The absence of helix 1 was also observed in the crystal structures of DAX-1 (25) and Rev-erbα and β (26). However, the expression constructs used in these structures did not include many residues before the N terminus of helix 3. The absence of helix 1 in the TR4 LBD structure is unexpected given the sequence homology between TR4, RXR, and COUP-TFII (supplemental Fig. S1), and it highlights the high degree of structural variation in the N termini of nuclear receptor LBDs.

![FIGURE 1. Crystal structure of the ligand-free TR4 LBD. A, the TR4 LBD dimer, showing that its dimer interface is formed predominantly by helix 10 (cyan). Helix 10 and its N and C termini are labeled. B, front and side views of the TR4 LBD monomer. The secondary structure assignment is labeled. The AF-2 helix is highlighted in red, and helix 10 is highlighted in cyan. C, the ligand binding pocket within the bottom half of the TR4 LBD is occupied by large hydrophobic side chains. D, the interaction interface between the TR4 AF-2 helix (pink) and the cofactor binding site of the receptor with the interaction residues labeled. E, overlay of the TR4 LBD structure and its AF2 helix (red) with the SRC-1 LXXLL motif (yellow) from the RXRα structure.]

| TABLE 1 | Statistics for crystallographic data collection and structure refinement |
|----------|---------------------------------------------------------------|
| Data collection | Resolution (Å) | 50–3.00 (3.11–3.00)* |
| | Space group | F222 |
| | Unit cell a, b, c (Å) | 129.48, 140.96, 184.86 |
| | Reflections, unique/total | 17576/128702 |
| | Completeness (%) | 99.8 (100) |
| | Rmerge (%) | 7.7 (85.7) |
| | Intensity, I/σ | 25.2 (2.3) |
| Structure determination and refinement | Resolution range (Å) | 30–3.0 |
| | Protein residues | 462 |
| | No. of water molecules | 73 |
| | R/Rfree (%) | 25.8/28.7 |
| | r.m.s.d.* bonds (Å) | 0.02 |
| | Angles (°) | 1.40 |
| | Mean B value (Å$^2$) | 72.4 |

*a Values in parentheses are for highest-resolution shell. 
*b r.m.s.d., root mean square deviation.
The second key feature is the absence of a ligand binding pocket at the lower part of the TR4 LBD. In this structure, the C-terminal part of helix 10 bends and collapses into the space where the ligand binding pocket should be (Fig. 1, B and C, and supplemental Fig. S3A). The bending starts from a kink at residues Met-572, Ser-573, and Ser-574 and makes the C-terminal part of helix 10 twist about 180°. The aromatic side chains of residues Phe-581 and Phe-582 of helix 10 point inward and form a large hydrophobic cluster with residues Leu-412 and Leu-413 from helix 3; Trp-442, Phe-446, and Leu-450 from helix 5; Met-457 and Leu-459 from the loop following helix 5; and Leu-494 from helix 7, thus totally blocking the ligand binding pocket (Fig. 1C).

The third key feature of the TR4 LBD structure is the occupation of the coactivator binding site by the AF-2 helix (Fig. 1, B and D, and supplemental Fig. S3B). The position of the AF-2 helix is orientated by the bending of the C-terminal part of helix 10, which pulls the small helix between helices 10 and AF-2 toward the core of the LBD structure and places the AF-2 helix at the coactivator binding site of the LBD (Fig. 1, B and D, and supplemental Fig. S3B). The AF-2 helix is stabilized in the coactivator binding site in two ways. The first is via a charge interaction between Glu-592 of helix AF-2 and Arg-438 from helix 4 of the coactivator binding cleft of the LBD (Fig. 1D). The second, and perhaps more important, is via the hydrophobic interaction of AF-2 residues Ile-591, Ile-595, Ile-598, and Leu-599 with the hydrophobic clusters formed by helices 3, 4, and 5 and the bent part of helix 10, thus forming a large extended hydrophobic region in the lower part of the LBD core structure (Fig. 1D). The sequence of the TR4 AF-2 helix, IXXIIXX, is highly related to both the coactivator LXXIIXX (or LXXIIX) and the co-repressor LXXXIXXXL (or LXXXIXXX) motifs (27). Based on structural superposition, the position of helix AF-2 at the coactivator binding site of the TR4 LBD closely resembles the position of the coactivator SRC1 motif at the RXRa coactivator binding site (Fig. 1E).

The occupation of the coactivator binding site by the AF-2 helix would block the binding of either a coactivator or a corepressor to the LBD and inhibit transcription activation by this receptor. This feature is the same as the one we observed in the ligand-free COUP-TFIi LBD structure (supplemental Fig. S2). Thus, the ligand-free TR4 LBD crystal structure we determined is in an autoinhibited inactive conformation similar to that of COUP-TFIi.

TR4 Serves as a Transcription Activator on Multiple Promoters—TR4 is expressed in numerous tissues, binds to direct repeat AGGTCA sequences in gene promoters, and regulates the expression of its target genes (9). To correlate the observed crystal structure with its functional activity of transcription regulation, we established a cell-based assay in the COS-7 cell line. We used a full-length TR4 expression construct and a luciferase reporter gene driven by promoters derived from two well-characterized TR4 target genes, oxytocin (28) and phosphoenolpyruvate carboxykinase (29), as well as from the COUP-TFIi target gene nerve growth factor inducible A (22). The oxytocin promoters that we used were the original promoter, DR0 (with a 0-nucleotide spacer), DR4 (with a 4-nucleotide spacer), and five repeats of DR0 separated by 4 nucleotides. TR4 was able to activate all these reporters in response to increased concentration of the TR4 expression constructs (Fig. 2A). The ability of TR4 to activate these DR0 and DR4 reporters is consistent with previous data (28, 29) and suggests that TR4 can function as a transcription activator.

The full-length TR4 is a 615-residue protein with three major domains: NTD, DBD, and LBD. To determine the contribution of each domain to the transcriptional activity of TR4, we performed cell-based assays with a series of deletion mutants (Fig. 2B). The results showed that both the NTD and the LBD are critical for transcriptional activity. The mutant with the LBD deleted lost all transcriptional activity, whereas the mutant with the NTD deleted lost about 90% relative to the activity of the wild-type receptor (Fig. 2B). This result indicated that the LBD is essential for ligand binding and coactivator recruitment and for its function as a transcription regulator.

To test the role of the AF-2 helix in TR4 transcription activation, we created a mutant with the AF-2 helix truncated at Val-589. This mutant had about 60% activity in the absence of coactivator and 40% activity in the presence of the coactivator PGCIα, relative to that of the wild-type receptor (Fig. 2C), indicating that an intact AF-2 helix is required for coactivator recruitment and transcription activation by this receptor.

TR4 Activation Requires the Formation of a Functional Dimer—The formation of a functional homodimer has been observed for many NRs, including RXRs (14, 30) and COUP-TFs (15, 31). We noticed that the TR4 LBD forms a dimer in solution when the protein is run through a size exclusion column (supplemental Fig. S4). We observed that the TR4 LBD forms a dimer in the crystal structure as well. The TR4 dimer in the crystal is stabilized by hydrophobic interactions between helices 10 and 7 and the loop region between helices 8 and 9 from the two opposite monomers (Fig. 3A). The interface residues of helix 10 are Ala-560, Leu-563, Val-564, Leu-566, Pro-567, Ala-568, Arg-570, and Leu-571, among which Leu-563, Leu-566, and Arg-570 are conserved among many NRs (supplemental Table S1, supplemental Fig. S1).

To determine whether or not dimer formation is required for transcription activation, we mutated two key conserved interface residues, Leu-563 and Arg-570, to alanine. Size exclusion chromatography demonstrated that the double mutant was monomeric in solution, indicating that the mutations of the conserved interface residues to alanine abrogate the dimerization in vitro (supplemental Fig. S4). A cell-based assay showed that each single mutation to alanine reduced transcriptional activity of this receptor by 40–50%, and the double mutant L563A/R570A retained only 30% of full activity relative to the wild-type receptor (Fig. 3B). These data indicate that the formation of a functional dimer is required for transcription activation by TR4, consistent with the previous observations that TR2 and TR4 form functional homodimers or heterodimers with each other (9).

**Activation of TR4 by Coactivators Requires the LXXLL Motifs**—Coactivator recruitment is a key step for transcription activation by NRs. We carried out a cell-based activation...
assay using the coactivators PGC1α and members of the steroid receptor coactivator family, SRC-1, SRC-2, and SRC-3. All coactivators significantly potentiated transcription activation by TR4, with PGC1α having the highest activity (Fig. 4A).

A nuclear receptor recruits a coactivator to a conserved coactivator binding cleft that binds to the leucine-rich LXXLL motif of the coactivator. The binding includes hydrophobic interactions between the hydrophobic residues in the cleft of the receptor LBD and the leucine-rich motif and interactions between the charge clamp residues of the NR LBD and the polar parts of the leucine-rich motif. Many NRs have two charge clamp residues, one positively charged (usually lysine) at the end of helix 3 of the NR LBD and one negatively charged (glutamic acid, or occasionally aspartic acid) at the center of the AF-2 helix of the LBD (32). Our recent studies showed that in the human androgen receptor, the positively charged residue is essential for binding to the coactivator motif and that the negatively charged one plays a supporting role (21).

Based on a sequence alignment of TR4 with RXRα (supplemental Fig. S1), the residues at the charge clamp positions of TR4 are Arg-411 at the end of helix 3 and Tyr-597 in helix AF-2. It is obvious that Tyr-597 is not able to form a charge clamp, although it might form hydrogen bonds and/or hydrophobic interactions with residues from the coactivator motif.

To test the role of the charge clamps in transcription activation by TR4, we mutated the presumed charge clamp residue Arg-411 to alanine and Tyr-597 to either alanine or glutamic acid. A cell-based assay showed that although the R411A mutation significantly reduced the TR4-mediated transcription activation, the Y597A or Y597E mutations had little effect (Fig. 4B), indicating that only Arg-411 plays an effective role in coactivator recruitment by TR4. This is consistent with our observation that the positive charge clamp residue is more important than the negative one for NR binding to a coactivator motif (21).

In contrast to the charge clamp, hydrophobic interaction is critical for coactivator recruitment by TR4. The mutation of hydrophobic residues Phe-414, Met-417, Val-438, or Ile-594/595 of TR4 to alanine significantly reduced TR4 activation (Fig. 4B). Furthermore, a mutant coactivator PGC1α with either of its LXXLL motifs mutated to LXXAA had 40–60% of the activity of the wild-type coactivator, whereas a mutant with both LXXLL motifs mutated to LXXAA retained only 20% activity (Fig. 4C). These results indicated that the hydrophobic interaction between the coactivator motif LXXLL and the hydrophobic coactivator binding cleft of the receptor is critical for coactivator recruitment and transcription activation by TR4.
The double mutant W442A/F446A retained only about 10% of the activity of the wild type (Fig. 5C). These results indicate that TR4 is a ligand-regulated receptor that requires an intact ligand binding pocket for ligand binding and transcription activation.

TR4 Is Ligand-regulated Nuclear Receptor and Is Activated by Retinol—Given that TR4 is a ligand-regulated nuclear receptor, we asked what ligands could induce TR4 transcription activation. Because TR4 has an LBD structure similar to those of COUP-TFII and RXRα, which are activated by retinoids (13, 15), it is reasonable to expect that TR4 ligands may be some type of retinoid. The active model of TR4 created by molecular modeling had a ligand binding pocket of 560 Å³ (Fig. 5, A and B), which would accommodate a retinoid compound. To identify potential TR4 ligands, we screened about 60 retinoid compounds using a luciferase reporter assay for their ability to promote TR4 transcription activation. Although many retinoids had little effect, a few significantly enhanced TR4-regulated transcription activation, among which retinol and all-trans-retinoic acid were the most potent (Fig. 6A). Interestingly, the level of TR4 activation by retinol was higher than that by atRA, indicating a difference in the ligand preference between TR4 and classic retinoic acid receptors, which prefer atRA.

Further experiments showed that the potencies of retinol and atRA on TR4-mediated transcription activation were dose-dependent (Fig. 6, A and B). Full dose curves determined by an AlphaScreen assay revealed that to promote TR4 to recruit the coactivator motif SRC3-3, the EC₅₀ values were 19.4 and 23.8 μM for retinol and atRA, respectively (Fig. 6B). Although the concentration of each retinoid required to activate TR4-mediated transcription was higher than physiological levels, those results were consistent with our mutagenesis data that TR4 is a ligand-regulated nuclear receptor and demonstrated that retinol and atRA are active for TR4-mediated transcription activation.

DISCUSSION

In this study, we determined a crystal structure of the ligand-free TR4 LBD, which revealed an autoinhibited conformation similar to that of the orphan receptor COUP-TFII (supplemental Fig. S2). Using a cell-based luciferase reporter assay, we demonstrated that TR4-regulated transcription was activated by retinol and all-trans-retinoic acid and was potentiated by coactivators including PGC1α and the members of the SRC family. These observations are consistent with our computer model showing that the active conformation of TR4 LBD has a ligand binding pocket of 560 Å³, suitable for accommodating a retinoid ligand. The orphan nuclear receptor TR4 is therefore a ligand-regulated receptor whose transcriptional activation requires an intact ligand binding pocket, the AF-2 motif, the dimer interface, and the coactivator binding site.

The Autorepressed TR4 LBD Conformation and Its Activation—The TR4 LBD structure in the absence of ligand adopts an autorepressed conformation with the coactivator binding site blocked by the AF-2 helix. This inactive conformation is facilitated by the collapse of the apo-ligand binding pocket, which is occupied by the bent C-terminal part of helix.
10. A similar autorepressed structure is also observed in several other apo-NR LBDs, including those of COUP-TFII (15), RXRs (33), DAX-1 (25), and Rev-erbs (26). Without a ligand in the pocket, the hydrophobic part of helix 10 is pushed by the surrounding solvent molecules toward the hydrophobic ligand binding pocket to form a more stable inactive LBD conformation. The bent part of helix 10 is twisted and flips about 180°, thus allowing the large hydrophobic residues of helix 10, Phe-581 and Phe-582 in TR4, to point inward and form close contacts with hydrophobic residues in the ligand binding pocket. In RXRα, ligand binding straightens the bent helix 10, rearranges the downstream loop region and helix AF-2, and converts the autorepressed structure into an active conformation for coactivator recruitment and transcription activation (14, 30, 34).

A straight helix 10 is also observed in ligand-bound Rev-erbβ LBD (35). Sequence analysis indicates that the C-terminal portion of helix 10 is rich in hydrophobic residues; thus, it ideally fits into the hydrophobic ligand binding pocket when there is no ligand (supplemental Fig. S1). Together, these structures suggest that the collapse of helix 10 into the ligand binding pocket of the apo-receptors is a general structural feature of these nuclear receptors.

**Vitamin A Signaling and Nuclear Receptors**—Members of nuclear receptor subfamily 2 (TR2/TR4, COUP-TFs, and RXRs) share a high degree of sequence similarity and identity in their ligand binding domains (supplemental Fig. S1). We previously identified that retinoic acids are activators for COUP-TFII (15), and in this study, we report that retinol and atRA are agonists for TR4. Together with the activation of
RXR by 9-cis-RA, these data suggest that the members of NR subfamily 2 share the same class of small molecule ligands and therefore can be defined as a group of retinoid-activated NRs. Our finding implies an expanded role for retinoids in signaling pathways mediated by these orphan nuclear receptors.

Retinoids are a class of derivatives of vitamin A, which is known to have great physiological importance for humans and animals. The parent compound of vitamin A is retinol, which can be reversibly oxidized to retinal, the active form in visual functions of the rods and cones of the retina. Retinal can be further oxidized irreversibly to retinoic acids, which are thought to be responsible for all the effects of vitamin A in morphogenesis and in growth and development through the binding and activation of the retinoic acid receptor (RAR) subfamily of nuclear receptors. However, there are many biological phenomena of vitamin A that cannot be explained by the action of retinoic acid (RA) and RAR alone. For example, retinol but not retinoic acid is required for feeder-free maintenance of embryonic stem cells (36). On the other hand, retinol, the parent compound of all retinoids, has never been shown to have direct biological functions through NR signal-
ing pathways. It is reported that retinol regulates bone morphogenetic protein-4 (BMP-4) expression in the male germ line, through the promoters of the BMP4 gene, for spermatogenesis in mice (37, 38). Interestingly, our data show that TR4 can be activated by retinol and retinoic acid with a slight preference for retinol (Fig. 6A). These data, together with previously reported results, suggest that the role of retinol in mammalian spermatogenesis may be through the activation of TR4 (39). Although the exact identity of the retinoids for the TR4 activation in a physiological setting remains to be discovered, our finding of retinol as a TR4 activator provides critical insight into a mechanism for the function of TR4 in mammalian spermatogenesis. Together with previous evidence for the activation of RXR and COUP-TFI by retinoids, the activation of TR4 by retinol presented in this study further indicates an expanded signaling network of vitamin A beyond the classic retinoic acid-RAR pathways.

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