Definition of the Surface in the Thyroid Hormone Receptor Ligand Binding Domain for Association as Homodimers and Heterodimers with Retinoid X Receptor*

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Thyroid hormone receptors (TRs) bind as homodimers or heterodimers with retinoid X receptors (RXRs) to DNA elements with diverse orientations of AGGTCA half-sites. We performed a comprehensive x-ray crystal structure-guided mutation analysis of the TR ligand binding domain (TR LBD) surface to map the functional interface for TR homodimers and heterodimers with RXR in the absence and/or in the presence of DNA. We also identified the molecular contacts in TR LBDs crystallized as dimers. The results show that crystal dimer contacts differ from those found in the functional studies. We found that identical TR LBD residues found in helices 10 and 11 are involved in TR homodimerization and heterodimerization with RXR. Moreover, the same TR LBD surface is operative for dimerization with direct repeats spaced by 4 base pairs (DR-4) and with the inverted palindrome spaced by 6 base pairs (F2), but not with TREpal (unspaced palindrome), where homodimers appear to be simply two monomers binding independently to DNA. We also demonstrate that interactions between the TR and RXR DNA binding domains stabilize TR-RXR heterodimers on DR-4. The dimer interface can be functional in the cell, because disruption of key residues impairs transcriptional activity of TRs mediated through association with RXR LBD linked to GAL4 DNA-binding domain.

Thyroid hormone receptors (TRs) are members of the nuclear receptor superfamily, which includes receptors for steroid hormones, vitamins, retinoids, prostaglandins, fatty acids, and orphan receptors for which no ligands are known (1–4). These receptors are modular transcription factors that bind to specific sequences in promoters of target genes. They bind DNA as monomers, homodimers, or heterodimers through the DNA binding domain (DBD) (1, 5). Steroid receptors bind as homodimers to two half-sites of a DNA palindrome. In contrast, TRs, retinoic acid receptors (RARs), vitamin D receptors (VDRs), peroxisome proliferator-activated receptors (PPARs), and several orphan receptors bind as heterodimers with retinoid X receptors (RXRs) to direct repeats (DRs) of the AGGTCA half-site (1) but also bind to elements oriented as palindromes or inverted palindromes (1). Binding specificity of these receptors is determined by spacing between the half-sites, because PPARs, VDRs, TRs, and RARs bind preferentially to DRs spaced by 1, 3, 4, or 5 nucleotides, respectively (1). Unlike PPARs, VDRs, and RARs, TRs also bind DNA as monomers and as homodimers to DRs and to inverted palindromes spaced by 4–6 nucleotides (F2) (1, 6, 7).

Current concepts of the dimerization and heterodimerization interfaces for nuclear receptors are based on mutational and x-ray crystal structural data. The mutation data for LBDs are mostly limited to TR, RAR, VDR, and RXR. Little is known from mutation studies with the steroid receptors, because only few studies report single mutations in the LBDs that disrupt dimerization (8). This is due in part to the fact that the LBDs and amino-terminal fragments of these receptors contribute substantially to stabilization of the dimer (9, 10).

Data for the TR, RAR, RXR, and VDR LBD dimerization have come from point mutations and deletions. These studies have identified an area encompassing about 40 amino acids in helices 10 and 11 (H10 and H11) that appears to mediate formation of RXR, and TR homodimers, and RAR-RXR, TR-RXR, and VDR-RXR heterodimers (11–15). However, this interpretation was based on results with mutations of residues placed in the interior core structure of the LBD, such as with the leucines in H10 and H11 in the so-called heptad repeats (16). Thus, these and the deletion mutations may disrupt receptor folding, complicate the interpretation of results, and, consequently, not allow for a definition of the actual interface or specific residues involved in forming homodimers or heterodimers.

Mutation data have also suggested that the region and residues within the TR LBD that form homodimers are distinct from those that form heterodimers (15). Current concepts of the dimerization interfaces for nuclear receptors are based on mutational and x-ray crystal structural data. The mutation data for LBDs are mostly limited to TR, RAR, VDR, and RXR. Little is known from mutation studies with the steroid receptors, because only few studies report single mutations in the LBDs that disrupt dimerization (8). This is due in part to the fact that the LBDs and amino-terminal fragments of these receptors contribute substantially to stabilization of the dimer (9, 10).

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from those that form heterodimers. For example, changing leucines to arginines in H11 of chicken TRα1 (L365R and L372R) or human (h) TRβ1 (L428R) disrupted TR-RXR heterodimers, but not TR homodimers on TREpal (the unspaced palindrome), DR-4 elements (direct repeat elements spaced by 4 base pairs), and inverted palindromes (11, 17). Furthermore, single mutations in arginine 316 to histidine, arginine 338 to tryptophan, and arginine 429 to glutamine in hTRβ1 disrupt homodimers but not heterodimers (18). Whereas these data indicate differences in requirements for heterodimerization and homodimerization, the usefulness of these mutations for defining the TR homodimerization or heterodimerization surface is limited, because these mutations are in the interior core structure of the TR.

Several investigators demonstrated that TR-RXR heterodimers interact in solution through a LBD heterodimer interface. It has been proposed that these heterodimers bind DR-4 elements by initial formation in solution of the LBD-LBD interface, which leads, upon DNA binding, to the formation of a second interface involving the second zinc finger of the RXR LBD and the first zinc finger of the TR LBD (19–22). Indeed, crystallographic studies of isolated TR-RXR DBDs bound to DR-4 supported the existence of a DBD heterodimer interface in which the RXR occupies the 5′ half-site, whereas TR occupies the 3′ half-site (23). However, there is no functional evidence that DBD surfaces participate in complexes formed by full-length TR homodimers or TR-RXR heterodimers bound to DR-4 or other elements. Also, if DBD surfaces participate in dimerization, it is unclear if the LBD and DBD surfaces form sequentially, nor is it known whether a LBD surface is used by TR homodimers to bind any DNA element. To date, no x-ray crystal structural data are available for TR LBD homodimers or heterodimers bound to DNA, and no comprehensive mutation analyses are available that locate the homodimer or heterodimer TR LBD interface.

RXRa (24), ERα (25), ERβ (26), PPARγ (27), and PR (28) LBDs crystallized as homodimers. Crystals of LBD heterodimers were recently reported for the RXR-RAR (29), where the interface is similar to that observed in the RXRa, ERα, ERβ, and PPARγ homodimers (24–27) and for the PPARγ-RXRa heterodimer (30), where the interface is slightly asymmetric with the PPAR LBD rotated ~10° relative to that expected from the C2 symmetry axis of the RXR LBD. The residues in H10 and proximal region of H11 form more than 75% of the total LBD dimer surface found in homodimers or heterodimers, whereas PR homodimers contacts are in the distal part of H11 and H12. The PR dimerization domain also covered a much smaller area (700 Å2) than that for the ERα (1703 Å2), which led the authors to question the physiological relevance of the observed dimer (31).

In the studies reported here we performed a comprehensive x-ray crystal structure-guided mutation analysis of the TR LBD surface to map the functional interface for TR homodimers and heterodimers with RXR in the absence and/or in the presence of DNA. This approach employed the x-ray crystal structures of the TR-RXR DBDs and TR LBD (16, 23) and was previously used by us for definition of a TR coactivator-binding surface (34). We also identified the molecular contacts in TR LBDs crystallized as dimers. The results show that crystal dimer contacts differ from those found in the functional studies. We found that identical TR LBD residues are involved in TR homodimerization and heterodimerization with RXR. Further, the same LBD surface is operative irrespective of the orientation and spacing of the half-sites for DR and inverted palindromic elements, but not for the TREpal homodimers, which appear to be simply two monomers binding to DNA. We also demonstrate that interactions between the DBDs stabilize TR-RXR heterodimers on DR-4. The dimer interface can be functional in the cell, because disruption of key residues impairs transcriptional activity of TRs mediated through association with RXR LBD linked to GAL4 DNA-binding domain.

**MATERIALS AND METHODS**

**Construction of TR Mutants**—TR mutants were created by ligating double-stranded oligonucleotides encoding the mutant sequence into the pCMX vector that encodes the full-length 461 amino acid hTRβ1 sequence. Some mutations were created using QuickChange site-directed mutagenesis kits (Stratagene). The mutated sequences were verified by DNA sequencing using Sequenase Kits (Stratagene). These TR mutants changed the amino acids in the hTRβ1 surface that were selected for scanning mutagenesis using the x-ray structure of the TRα LBD as a guide to localize the surface residues on hTRβ1 (16, 34).

Subsequent solution of the hTRβ1 LBD structure (35) revealed that the mutations were placed correctly. Most mutations changed the resident amino acid to arginine, reasoning that bulky and charged residues on the surface were more likely to disrupt TR association with other proteins over a wider range without reducing TR solubility. Each mutant maintained the overall integrity of TR as judged by the ability to bind [3H]T3, with Kd values between 4 and 174% of that for the native receptor and/or to bind the glucocorticoid receptor interacting protein 1 (GRIP1) (not shown) as effectively as the wild-type TR. We initially tested, as reported previously, widely separated locations to probe the entire LBD surface for dimerization (34). Subsequently, a series of mutations were performed along H10 and H11 in a region where residues were identified that disrupted dimerization to define better the structural requirements for dimerization. Mutations are represented by the single-letter amino acid abbreviation of the native residue followed by its position number in the sequence and the mutated residue abbreviation. The affinity of the wild-type TRβ1 is 60 ± 7 pm.

The relative affinity was determined by dividing the wild type by the mutant Kd. The 62 mutants included and their relative affinities are:

- E217R (91%), W219K (not done (ND)), E227R (109%), R242E (68%), F245K (ND), D251R (ND), V256R (ND), E257R (87%), H271R (91%), T277R (7%), T281R (94%), V284R (105%), D285A (68%), K288A (61%), M292K (ND), C294K (94%), E295R (150%), C298A (65%), C299R (44%), E299A (139%), I302A (91%), 1302R (99%), L305I (ND), K306A (5%), K306F (7%), C309A (ND), V319K (ND), E324R (ND), M334K (ND), V348R (ND), D351R (ND), M358A (ND), D355A (ND), M358A (ND), D358R (80%), P359R (121%), A363R (80%), V369A (59%), E390R (140%), E391R (109%), L409R (70%), H413R (81%), V414K (ND), H416R (113%), P419R (152%), L422R (68%), M423R (174%), T426R (48%), R429A (55%), M430R (187%), A433R (ND), C434R (124%), S437R (65%), L440R (129%), V444R (66%), T448R (282%), E449R (26%), F453E (32%), L454R (12%), L456R (13%), E457K (38%), V458M (ND).

A TR DBD mutant was created by changing the Asp177, known to interact with RXR in crystallographic studies, to Ala. A RXR DBD mutant was made by changing all Arg residues previously shown in crystallographic studies to be involved in dimerization with the TR to Ala (R172A, R182A, and R186A, Ref. 23). We also found that this RXR mutant had three additional DBD changes (T168P, T270I, and Y93A) that did not interfere with RXR binding to DNA. An RXR LBD mutant was made by changing leucines 419 and 420 into arginines, positions analogous to TR mutants defective in dimerization (see "Results").
Crystallography

**hTRβ—Crystallographic conditions for hTRβ LBD with Triac has been recently described fully (35). Briefly, crystals were transferred into a cryoprotectant composed of 30% glycerol, 1.4 M NaAc, and 100 mM NaCac, pH 7.2, and processed using HKL. A molecular replacement solution was found using AMORE from the space group P3121 (a = 68.9 Å, c = 131.5 Å) and contained 1 molecule of TR LBD. The N-terminal His tag was not removed prior to crystallization.**

**rTRα—Hexagonal crystals of rTRα LBD with T3 were obtained using hanging drop vapor diffusion at 17 °C from a drop containing a 1:1 mixture of 10 mM HEPES, pH 7.0, 0.5 M MgCl2, 0.5 mM EDTA, 20 mM NaCl, 5% glycerol, 0.1% monothioglycerol.**

**rTRα LBD solution at 20°C was cryoprotected using 28% glycerol, 1.4 M NaAc, and 100 mM sodium cacodylate, pH 7.2. Crystals were of space group P3121 (a = 108.64 Å, b = 110.25 Å, c = 133.72 Å).**

**Models of the TR-RXR Heterodimer**

These models were derived using the known structures of the TR (27) and the hRARα:mRXRα heterodimer (Ref. 29, Protein Data Bank (PDB) entry 1DKF). To create the model of the TR-RXR heterodimer, the rTR LBD was superimposed on hRAR using the Cα atoms of the hydrophobic core helices H3-H5-H6 (rTR residues Ile299–Arg326; hRAR residues Ile326–Arg356) and H9 (rTR residues Asp351–Leu352; hRAR residues Asp352–Met353), using LSQMAN (m.r.s. deviation of 0.9 Å for Ca; r.m.s. deviation of 1.2 Å for 203 Cα atoms after optimization). Hydrophobic and ionic surface residues in the crystallographic contacts were calculated using ASC, with a 1.4 Å probe (35, 39). Hydrogen bonds were identified using HBPlus (40).

**RESULTS**

**TR LBDs Form Crystals with Diverse Intermolecular Contacts—Crystal forms of LBD fragments of the TRα and TRβ subtypes exhibit two distinct symmetrical intermolecular contacts. In TRα, the individual monomers are rotated 180° with respect to each other, with an axis of symmetry parallel to H11, such that the helices cross with the junction near the mid-point of H11 (Fig. 1A). The contact buries a modest 550 Å² of surface area per monomer. The primary contacts are between residues in H11, with contributions from the C-terminal portion of H8. A cluster of hydrophobic residues in H11 marks the center of symmetry: Met376, Ile377, Ala378, and Cys379. At the periphery of the contact are electrostatic interactions between residues in H8 and H11: Lys386 with Asp332 and Glu331 with Asp377.**

**In TRβ, the individual monomers are again rotated 180° with respect to each other, but the axis of symmetry is perpendicular to H11, producing an antiparallel LBD dimer (Fig. 1B, Ref. 35). Rather than crossing as in the TRα, H11 of one monomer nestles between H8 and H11 of the other monomer. The contact buries 580 Å² of surface area per monomer. The residues in the contact again involve H11 but with the opposite end of H8. Hydrophobic contacts in H8 pack Val348 against Val348 and Ala349 in the other monomer. Hydrophobic contacts in H11 are made by Met429 and the aliphatic stem of His432 and between the hydrophobic cluster Met430, Ala431, and Cys432, just as in the TRα. Electrostatic interactions link Arg443 from strand S4, Lys442 in H7, and Asp455 in H8; and also Asp427 and His441 in H11, with an additional hydrogen bond between Asp377 and Ser387.**

**Scanning Mutations Define a LBD Interface for TR-RXR Association in Solution—Because the hydrophobic contacts described above may reflect forces operative during crystallization and not represent a functional dimerization surface, we mapped the TR LBD surface that participates in dimer formation by mutating the TR LBD surface. These mutations were introduced ~7 Å apart to scan the TR LBD area involved in protein-protein interactions (34). As described under the “Materials and Methods,” these mutations generally maintained overall receptor integrity and functions largely unrelated to dimer or heterodimer formation such as ligand and coactivator binding.**

**Sites important for TR-RXR interactions were first deter-
FIG. 1. Ribbon drawings of TR LBD homodimers. A, hTRα and B, hTRβ (35). To facilitate comparison of the homodimers, the left monomer in each appears in identical orientation (i.e., viewed down H10). The helices discussed in the text are labeled: H8 (blue-green), H10 (yellow), H11 (dark blue), and H12 (magenta).

These results demonstrate that the essential residues required for forming TR LBD homodimers with DR-4 or F2 are Leu400 in H10 and Leu422 and Met430 in H11. However, a larger group of residues flanking this critical core of amino acids is required for forming TR homodimers with DR-4 compared with F2.

We also asked if the surface used for TR homodimers on DR-4 and F2 and for TR-RXR heterodimers in solution is also operative for forming TR homodimers on TREpal. None of these mutants (Fig. 3C) or other surface TR mutants tested (data not shown) blocked formation of TR homodimers on TREpal. These data suggest that TRs bound to TREpal are simply two monomers rather than a homodimer.

Taken together, these results show that the TR LBD surface involved in formation of TR-RXR heterodimers in solution and TR homodimers with DR-4 or F2 is similar and is not involved in TRs bound to TREpal. This surface contains a critical core of hydrophobic amino acids comprised of Leu400 in H10 and Leu422 and Met430 in H11 that are required for all types of dimeric interactions. In H11, the residues flanking Leu422 and Met430 appear to be important for homodimers on DR-4 and for heterodimers in solution. These findings suggest that the dimer surface is centered on a set of critical hydrophobic residues, but that it is flexible and extends to adjacent residues (Fig. 3D).

The Same LBD Interface Mediates Formation of TR Homodimers and TR-RXR Heterodimers on DNA, but Heterodimers Are More Stable because of DBD-DBD Interactions—The mutations were examined for effects on TR-RXR heterodimer formation on DNA. None of the TR LBD single mutants that impaired formation of TR homodimers on DR-4 (see Fig. 3A) or an additional double mutant L422R/M423R containing two residues on the dimerization surface (data not shown) disrupted TR-RXR heterodimers on DR-4. However, L422R weakened formation of TR-RXR heterodimers on F2 (Fig. 3B, lane 16) and abolished them on TREpal (Fig. 3C, lane 16). Because Leu422 in the center of the surface is critical for TR-RXR heterodimers in solution and TR homodimers on DR-4 and F2, it is likely that the TR-RXR heterodimer surface on F2, TREpal, and even DR-4 is also similar but that TR-RXR heterodimers are more stable.

Because inverted palindrome DNA sequences have the highest capacity for binding TR homodimers (6), we used the F2 palindrome to examine whether the TR LBD surface that forms homodimers on F2 coincides with that mapped for homodimers on DR-4 and for TR-RXR heterodimers in solution. Fig. 3B shows that this is the case because L400R in H10 and L422R and M423R in H11 (lanes 7, 15, and 17) disrupted homodimers with F2. However, in contrast to DR-4, the E393R and P419R mutants (lanes 5 and 13) partially disrupt homodimers on F2.

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The LBD Surface That Forms TR-RXR Heterodimers in Solution also Forms TR Homodimers on DR-4 and F2 but Not on TREpal—Because homodimers with full-length TRs are not observed in solution, we next sought to define the TR surface used by TR homodimers on DNA. We first used the DR-4 binding site, because this is the most commonly described TRE. As shown in Fig. 3A, we found that a subset of the mutants that inhibited formation of TR-RXR heterodimers in solution also blocked TR homodimer formation on DR-4. Thus, E393R and L400R in H10 (lanes 5 and 7, respectively) and P419R, L422R, and M423R in H11 (lanes 13, 15, and 17) nearly abolished formation of TR homodimers on DR-4. The mutants T426R and M430R in H11 that impaired formation of TR-RXR heterodimers in solution had minimal influences on TR homodimer formation (Fig. 3A, lanes 19 and 21). No other TR mutants of the 50 tested spanning helices 1, 3, 5, 8, 9, and 12 were defective in homodimer assembly on DR-4 (data not shown).

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The greater stability of TR-RXR heterodimers on DR-4 could be in part because of DBD-DBD contacts as observed in the x-ray crystal structure of TR-RXR isolated DBDs (without LBDs) on DR-4 (23). However, the greater resistance to mutational disruption of the DR-4 bound TR-RXR heterodimers as compared with TR-TR homodimers could be because of the fact that only the TR subunit of the heterodimer pair contained a mutation whereas with TR-TR homodimers both subunits are

mined by examining binding of TRs to GST-RXR in pull-down assays. Wild-type hTRβ bound to GST-RXR in a T3-independent manner (Fig. 2A), whereas binding of wild-type or mutant TRs to GST alone was negligible (data not shown). Replacing amino acids in H10 (Glu400 and Leu405) and H11 (Pro415, Leu422, Met423, Thr426, and Met430) with Arg greatly diminished TR interactions with RXR (Fig. 2A). This was not observed with the mutants E390R in H10 and H416R and C434R in H11 (Fig. 2A) or 62 other mutants described under “Materials and Methods” and spanning helices 1, 3, 5, 8, 9, and 12 (data not shown). Fig. 2B shows the location of the amino acids involved for TR association with RXR in solution, from the GST-RXR interactions with TR mutants. All of the TR mutants defective in forming heterodimers with GST-RXR bound T3 and interacted with GST-GRIP1 as well as the wild-type TR (data not shown). Taken together, these results define a relatively small surface in H10 and in the amino-terminal portion of H11 of the TR LBD that is necessary for binding RXR in solution.

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mutated. To address this issue, we assayed TR-RXR heterodimer formation where both the TR and RXR subunits are mutated. For these experiments, we replaced two leucines, Leu419 and Leu420, with arginines in the RXR LBD, which are residues analogous to the TR double mutant L422R/M423R that did not disrupt TR-RXR heterodimers on DR-4. As shown in Fig. 4A, the RXR L419R/L420R mutant markedly inhibited heterodimer formation with wild-type TR on DR-4 (lane 4). Mixing RXR L419R/L420R with TR L422R (Fig. 4A, lane 8) or L422R/M423R (data not shown) did not further disrupt heterodimers on DR-4. These findings indicate that those mutations in the LBDs of both subunits can reduce heterodimer formation on DR-4 and suggest that the RXR LBD dimer interface is similar to the one in the TR LBD. These findings together with the known structures of the TR (27) and the hRARα:RXRα heterodimer (Ref. 29, PDB entry 1DKF)...

Fig. 2. RXR binding by hTRβ1 wild type and mutants. A, in vitro binding of 35S-labeled hTRβ1 wild type and mutants to GST-RXRα (18). The binding assay was analyzed by autoradiography after separation using 10% SDS-polyacrylamide gel electrophoresis. The mutations with their helix locations are indicated above the gels. On the top we indicate the Input (40% of the amount used in the reaction) of each TR and on the bottom we show the TR binding to GST-RXRα. B, scheme showing the hTRβ LBD structure with α-helices drawn as ribbons and β-strands as arrows depicting the residues Glu393 and Leu400 in H10 (green) and Pro419, Leu422, Met423, Thr426, and Met430 in H11 (dark blue) inferred by mutations to form heterodimers with RXR in solution.

Fig. 3. Formation of homodimers or heterodimers of wild-type hTRβ1 or mutants on diverse TREs. Gel shift assays contained 40 fmols of the in vitro-translated 35S-labeled TRs (usually 1–3 μl), 3 μl of either unprogrammed lysate or unlabeled hRXRα and 10 ng of DR-4 (A) or F2 (B) or 2 ng of TREpal (C). The mutations with their putative helix locations are indicated above the gels. D, the surface of the hTRβ LBD is shown indicating H10 and H11 and the residues proposed to contribute to the dimer surface. Areas in the helices are colored according to their importance for dimerization. The surface represented in red forms an area surrounded by the hydrophobic amino acids Leu400, Pro419, Leu422, and Met430; the surfaces in green and blue represent adjacent residues that are progressively less important for formation of TR homodimers on DR-4 and F2.
allowed us to propose a model for the TR-RXR heterodimer, which is shown in Fig. 4B, depicting the major interactions between the hydrophobic residues in H10 and H11 of TR with H11 of RXR.

We also investigated if DBD-DBD interactions participate in TR-RXR heterodimers on DR-4 by using TR and RXR DBD mutations based on the crystal structure of the TR-RXR DBD dimers (23). The TR DBD mutant replaced the aspartic acid 177 with alanine and the RXR DBD mutant replaced the three arginines 172, 182, and 186 with alanines. The TR D177A formed efficient homodimers and heterodimers with either the wild-type RXR or the RXR DBD mutant on DR-4 (Fig. 4A, compare lanes 1–3 to 9–11) indicating that these DBD mutations per se do not disrupt formation of homodimers or heterodimers on DR-4. In contrast, heterodimer formation was completely abolished by mixing the TR D177A with the RXR L419R/L420R LBD mutant (Fig. 4A, lane 12). Similarly, TR-RXR heterodimers did not form (Fig. 4A, lane 7) when the RXR DBD mutant was mixed with the TR LBD mutants L422R (that formed efficient heterodimers with the wild-type RXR; Figs. 3A, lane 16 and 4A, lane 6) or L422R/M423R (data not shown). By contrast, the RXR DBD mutant did not disrupt heterodimers with L422R on the F2 DNA (data not shown). Collectively, the data show that LBD interactions are dominant in formation of TR homodimers and heterodimers on DR-4 because disruptions in LBD, but not in DBD interactions, impair TR dimer formation. These results also indicate that DBD interactions stabilize TR-RXR heterodimers on DR-4, because DBD mutations can enhance the effects of LBD mutations. The data provide additional evidence that the LBD surfaces that form TR-RXR heterodimers on DR-4 are similar to those utilized by TR-RXR heterodimers in solution and by TR homodimers on DR-4.

Mutation of TR Hydrophobic Residues in Helix 11 Impairs Transactivation in Vivo When RXR Is Linked to a Heterologous DBD—The TR LBD single mutants that impair assembly of TR homodimers on DNA and TR-RXR heterodimers in solution were assessed for their abilities to induce ligand-mediated transcription in cultured cells in a context where the interactions occur only through the LBDs, and the TR is not bound to DNA. We cotransfected GAL-RXR with TRs in CV-1 cells. In the presence of wild-type TR or any of the mutants that failed to impair the TR-RXR association in solution (E390R, H416R), T3 stimulated 25- to 48-fold the reporter gene driven by five GAL binding sites. In contrast, transcriptional activity induced by either P419R or L422R mutants was completely absent in this context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively. By contrast, the TR mutants T426R and M430R, which impaired activation relative to the wild-type TR, respectively. This context, and E393R and L400R displayed 10 and 35% activation relative to the wild-type TR, respectively.
tions affecting dimerization and/or heterodimerization retained other receptor functions such as ligand and coactivator binding. An additional advantage of the surface scanning approach is that analyses of multiple mutations demonstrate patterns and minimize erroneous conclusions derived from interpretations of results from a single mutant. These functional data can also determine whether the way receptors crystallize with purified proteins reflects the functional interactions in other conditions. Thus, we compared the interfaces found in two TR LBDs that crystallized as dimers with those determined from the functional studies. The data provide the first comprehensive analysis directed at mapping the LBD interface for forming TR homodimers and heterodimers with RXR. They suggest that a similar surface participates in formation of TR homodimers with DR-4 and F2 DNA and TR-RXR heterodimers in solution and with DR-4, F2, and TREpal.

The TR LBD crystals were obtained with both the rTrα and hTrβ LBDs. In both structures, the TR monomers were rotated 180° with respect to each other. With the rTrα, the helices crossed with a junction near the middle of H11, whereas with the hTrβ, the axis of symmetry is perpendicular to H11, producing an antiparallel LBD dimer in which one monomer lies between the end of H8 (opposite to that with the rTrα) and H11 of the other monomer. The contacts bury a modest 550 Å² and 700 Å² of solvent-exposed area per monomer for the rTrα and hTrβ LBDs, respectively. These observations could imply a weak interaction because the bona fide protein-protein interface typically buries > 700 Å² of solvent-exposed area (41). In support of this notion, none of the mutations of residues of either H8 or H11 involved in these contacts, such as Asp³⁵⁵, Ala⁴³³, Cys⁴³⁴, and Ser⁴³⁷, disrupted TR-TR dimer formation on DNA, whereas mutations of other residues of H10 and H11 did affect these interactions. These observations, taken together, suggest that the contacts observed in the TR crystal structures are not involved in formation of TR-TR homodimers on DNA and also differ from the contacts reported in the structures of RXR and PR homodimers (24, 26).

The scanning mutations were used first to define a TR-RXR LBD heterodimer interface when the TRs are soluble and RXRs are linked to GST (Fig. 2A). This method likely reflects interactions of the two receptor types in solution. TR homodimer interactions were not tested in this context, because they have not been observed in solution. Mutations in the TR LBD that blocked GST-RXR-TR interactions were located on a small surface comprising H10 and H11, (Fig. 2, A and B), whereas 30 mutations on other parts of the TR LBD surface failed to inhibit the reaction. Although this heterodimer surface differed from that observed in our TR-TR LBD homodimer crystals or of the PR homodimer (28), it resembles that observed in the structures for the RXXα (24), the ERα (25), the ERβ (26), and the PPARγ (27) homodimers. In other studies the functional protein-protein interfaces derived from mutational data have been shown to differ from those that form in the crystal. For example, about 30 side chains from growth hormone and its receptors make contact, but individual replacement of contact residues with alanine showed that only a central hydrophobic region accounts for more than three-quarters of the binding free energy (42). Furthermore, mutations in interface residues cause structural changes that redistribute the particular contributions of individual residues to the free energy of binding.

Interestingly, the hydrophobicity of residues central to the TR homodimer and TR-RXR heterodimer interactions, Leu⁴⁲² and Met⁴⁵⁵, is conserved in all the receptors, which exhibit that interaction in the crystal (RXXα (24), ERα (25), ERβ (26), PPARγ (27)). By contrast, in the PR and the other steroid receptors with the exception of ER, these residues are hydrophilic or charged, resembling the mutations introduced in the TR that disrupt the interaction. Thus, the PR homodimer interaction could be representative of one class of LBD interactions, the classic “homodimer” receptors, whereas the interactions examined here are representative of the “heterodimer” receptors.

Data in support of the notion that the TR utilizes the same LBD surface for forming homodimers with DR-4 and F2 DNA as it uses for heterodimer formation on GST is based on the observation that some of the same mutations in this surface disrupted all of these interactions. Variations in the effects of the mutations in the three contexts were observed, and some of the mutations that affected the TR-RXR-GST interaction failed to affect the homodimer interactions. However, the overall pattern suggests at the least an overlapping surface, and the same core of critical hydrophobic residues is employed by TR to form homodimers and heterodimers with RXR. This pattern is similar to the one found in the functional interface of growth hormone-growth hormone receptor where a small and complementary set of contact hydrophobic residues maintains binding affinity, a property that may be general to protein-protein interfaces (42). The surface centers on the Leu⁴²² residue in TR H11, which disrupted TR homodimers on DNA and TR heterodimers formed in solution. Whereas the overall surface ex-
tends from residues Pro
\textsuperscript{419} to Met
\textsuperscript{430} in H11 and from Glu
\textsuperscript{393} to Leu
\textsuperscript{400} in H10 (Fig. 2B), the individual contribution of these residues varies depending on the context in which the interactions take place. Thus, for TR homodimers bound to F2, only Leu
\textsuperscript{400} in H10 and Leu
\textsuperscript{422} and Met
\textsuperscript{423} in H11 appear to be the critical residues under the conditions studied, whereas flanking residues such as Glu
\textsuperscript{393} in H10 or Pro
\textsuperscript{419} in H11 are critical for TR homodimer formation on DR-4. Finally, an even larger set of flanking residues such as Thr
\textsuperscript{426} and Met
\textsuperscript{430} are critical for TR-RXR heterodimers in solution. That similar homodimerizing and heterodimerizing interfaces are used for TR LBDs to bind to DR-4 and F2 elements provides support for the model that LBD dimer interfaces are independent of orientation of TRE half-sites. Another interesting point derived from these studies is that contrary to the prevailing model, the receptors do not need to form dimers in solution before DNA binding. Thus, TR mutants incapable of interacting with RXR in solution, such as E393R, L400R, P419R, M423R, T426R, and M430R formed stable TR-RXR heterodimers with DNA.

Whereas mutations readily blocked TR binding to DR-4 and F2 elements, no mutation, including L422R, disrupted homodimers on TREpal. These data suggest that the “dimers” on TREpal consist of independent binding of two monomers. This notion is supported by the observation that “homodimers” on TREpal are formed very weakly as compared with those on DR-4 and F2 (6, 7). Another indication that TR “dimers” on TREpal are really two monomers is based on the fact that T
\textsubscript{3} increases TR binding as monomers with several TREs and as “homodimers” on TREpal (6, 7), whereas T
\textsubscript{3} decreases TR binding as homodimers with DR-4 or F2 elements (6, 7). The dimeric interactions on DR-4 and F2 and heterodimerization on GST-RXR also mediates TR-RXR heterodimerization on F2, F2, and TREpal elements. The first evidence supporting this conclusion is that L422R in the center of the surface weakens formation of TR-RXR heterodimers on F2 (Fig. 3B, lane 16) and abolishes them on TREpal (Fig. 3C, lane 16).

The finding that none of the TR LBD single mutants blocked TR association with wild-type RXR with DR-4 suggested the possibility that DNA may be stabilizing such interactions. This is supported by the observation that heterodimerization on DR-4 was impaired when the TR L422R mutant was incubated with the RXR DBD mutant. These findings argue that interactions among TR and RXR DBDs play a role stabilizing heterodimer formation on DR-4 and indicate a dominant role for the LBDs relative to DBDs in stabilization of both homodimers and heterodimers on DR-4. Our results further suggest that the analogous surface on the RXR mediates the interaction of this receptor with the TR. Thus, a double mutation in the RXR LBD, L419R/L420R greatly reduced formation of heterodimers with wild-type TR on DR-4. An analogous double mutation on the TR LBD, L422R/L423R, did not affect heterodimer formation with DR-4 to the same extent (data not shown), suggesting that RXR has a more dominant role in TR-RXR heterodimer formation and explain why, in contrast to TR homodimers, TR-RXR heterodimers are formed in solution and are not disrupted by ligand binding.

The formation of TR homodimers on DR-4 might be because of DBD-DBD interactions, or might simply result from placement of the LBDs together after independent binding of two receptor monomers to DNA. Our findings that isolated TR LBD mutations inhibited TR homodimers, and that TR DBD mutations did not, suggest that DBD-DBD interactions in TR homodimers, if they exist, are not functionally important for processes requiring dimers. These observations are consistent with results of other investigators that failed to detect formation of TR DBD dimers on DR-4 (20). If the DBDs do not interact in the TR homodimer, this could explain the greater stability of the TR-RXR heterodimer on DR-4. Interestingly, TR-RXR heterodimers are not stabilized by such DBD-DBD interactions on F2, because, in contrast to what is seen on DR-4, the weakened interaction between the TR L422R and wild-type RXR on F2 is not further disrupted by mixing the TR L422R with the RXR DBD mutant (data not shown). However, TR-RXR heterodimers do form on TREpal, where they appear to be weaker than the ones formed on F2 and DR-4, because L422R disrupts them completely. We interpret that in the case of TREpal, TR monomers recruit RXR using the same LBD dimer interface it utilizes to bind RXR in DR-4 and F2, but that the resultant TR-RXR complex bound to TREpal is less stable. RXR serves as a heterodimer partner to VDR and RAR and it was predicted that DBD-DBD contacts would stabilize VDR-RXR and RAR-RXR heterodimers (23). However, we previously found that a single LBD mutant in the VDR analogous to the L422R in the TR impaired the formation of heterodimers with RXR on DR-3 (45) suggesting that DBD-DBD contacts do not contribute to VDR-RXR heterodimers to the extent that they do with the TR-RXR heterodimers. It is noteworthy in this respect that the DR-4 configuration places the center of the DBDs with a 10 bp separation, which is approximately one turn of the DNA (10.5 bp) and aligns the DBDs in a way that could optimize contacts between the DBDs. It is possible that the shorter separation and 36° rotation expected between the RXR and the VDR twists the orientation of the DBDs to eliminate direct contact. In this context, VDR does not form homodimers on DNA (45). This again points to properties of the RXR that are dominant in forming the complex.

The current studies argue that TRs use a similar LBD surface for homodimerization and heterodimerization. However, there may be differences in the contacts made, even though they involve overlapping surfaces. In this regard, it is known that proteins may possess consensus sites with favorable intrinsic physiochemical properties that are dominant for protein-protein interactions with multiple partners (46). Modest adjustments occur within this flexible consensus site to accommodate binding to different partners and the crucial properties for this convergent binding surface are its accessibility, hydrophobicity, and limited charge interactions at its periphery (46). Such properties are indeed analogous to the ones we found in the dimer surface of TR and RXR, where a patch of hydrophobic residues are readily accessible for protein interactions. Crystal structures of heterodimeric complexes of RARα and RXRα LBDs (29) and PPARγ and RXRα LBDs (30) revealed that the helices of these receptors, which correspond to H10 and H11 of TR, contribute more than 75% of the dimer surface. A hydrophobic core of residues analogous to the ones described here for the TR-RXR LBD interactions is at their centers. Our model for the heterodimeric interface of TR and RXR LBDs based on functional studies closely matches the surfaces reported for the crystal structures of RARα-RXRα and PPARγ-RXRα LBD heterodimers. This finding supports the contention that the conservation of interface residues of this subgroup of nuclear receptors, which is significantly higher than that of the entire LBD, enables these receptors to engage in heterodimer formation with RXR (29) and predicts that the LBD surfaces for heterodimerization are likely to be similar within this subgroup of nuclear receptors.

We addressed whether the mutations could block functional receptor-receptor interactions in vivo, by using a heterologous system that employed the GAL-DBD linked to the RXR LBD and TR mutants with a reporter gene containing GAL DNA binding sites. Several mutants that blocked formation of
In summary, the data define a common domain of the TR and RXR that is involved in TR-RXR dimerization and TR-RXR heterodimerization. The strength of the interaction varies and can be influenced by the partner, DNA, DBD-DBD interactions and probably other proteins that form higher order complexes in the cell. In some, but not all cases, interactions between this domain on the TR are important for TR action.

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Definition of the Surface in the Thyroid Hormone Receptor Ligand Binding Domain for Association as Homodimers and Heterodimers with Retinoid X Receptor
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