Ligand-binding and heterodimerization activities of a conserved region in the ligand-binding domain of the thyroid hormone receptor

(thyroid hormone receptor auxiliary protein(s)/heptad repeats/amphipathic α-helix/protein–protein interactions/transactivation)

REMCO A. SPANJAARD*, DOUGLAS S. DARLING, AND WILLIAM W. CHIN

Division of Genetics, Department of Medicine, Brigham and Women’s Hospital, and Howard Hughes Medical Institute and Harvard Medical School, Boston, MA 02115

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ABSTRACT The ligand-binding domain of the thyroid hormone (3,5,3'-triiodothyronine) receptor (TR) contains poorly characterized subdomains involved with ligand binding, transactivation, and protein–protein interactions. The region between residues 288–331 of rat TRα-1 was analyzed by modeling and site-directed mutagenesis. Our results suggest that part of this sequence adopts an amphipathic α-helical conformation. The integrity of the putative helix is important for 3,5,3'-triiodothyronine binding but not necessarily for heterodimerization with nuclear factor(s). Mutants defective for both activities were found clustered in a region overlapping the C-terminal portion of the helix and further downstream. The sequence conservation of this particular region among the entire superfamily suggests a similar role in dimerization in other receptors.

Thyroid hormone (3,5,3'-triiodothyronine; T3) receptors (TR) are members of the nuclear hormone receptor superfamily that are homologous, especially in the DNA-binding region. More complicated and less well-conserved is the C terminus, or ligand-binding domain (LBD), where several functional subdomains can be recognized that subserve ligand binding, ligand-dependent transactivation, and protein–protein interactions (1, 2). At present, the precise nature of these subdomains is largely unknown.

We have examined a region in the LBD of rat TRα-1 (rTRα-1) located between residues 289–318 and found evidence that part of this region adopts an amphipathic α-helical conformation important for T3 binding and heterodimerization with nuclear TR auxiliary protein(s) (TRAP) (3–5). Sequences downstream of this putative helix also contribute to these activities.

MATERIALS AND METHODS

Expression Vectors for rTRα-1 and Point Mutants. rTRα-1 cDNA (6) was modified by creation of unique HindIII and BamHI sites 3' next to the stop codon (unpublished results). cDNA was digested with EcoRI and BamHI and cloned into the EcoRI and BglII sites of pSG3X, a derivative of pSGS (Stratagene), giving pSGTrα-1 or clone 1 (wild type). Standard cloning procedures were followed (7). The identity of each construct was verified by DNA sequencing.

Site-Directed Mutagenesis. The Xba I–HindIII fragment of pSGTrα-1, which contains the LBD, was cloned into M13mp18 and used to generate single-stranded DNA in Escherichia coli K-12 strain CJ236 (dom, ung, thi, relA; pCJ105 (Cm')3), which served as a template for all oligonucleotide-directed mutagenesis (Bio-Rad). Mutations were confirmed by sequencing and were numbered 2–16. Mutated fragments were cut out with Xba I–HindIII and used to replace the wild-type Xba I–HindIII fragment. Subcloning of mutations was verified by sequencing. Oligonucleotide sequences can be obtained on request.

In Vitro Transcription, Translation, and T3 Binding. Plasmids were linearized with HindIII and transcribed with T7 RNA polymerase (8). mRNA was translated in rabbit reticulocyte lysate (Bethesda Research Laboratories) in the presence of [35S]methionine (Amersham). Incorporation of [35S]methionine into protein was monitored by trichloroacetic acid precipitation (9). Five microliters of either unprogrammed or programmed reticulocyte lysate was used for binding to 0.5 nM [125I]-labeled T3 (81.4 GBq/mmol, NEN), and specific binding was determined as described (8).

Transfections. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium/10% fetal calf serum and were transfected in 6-cm plates by using the DEAE-dextran method (7). Cells were then grown for 48 hr with or without T3 (100 nM) before harvesting. The same extract was used for luciferase (8) and chloramphenicol acetyltransferase (CAT) activity determinations (10). Conversion of [14C]chloramphenicol (2.2 GBq/mmol, NEN) was determined by direct scanning of TLC plates with a Betascope 603 analyzer (Betagen).

DNA Binding/Heterodimerization Assay. The avidin–biotin complex DNA-binding (ABCD) assay used to determine the binding of in vitro-translated (mutant) receptors to the biotinylated rat pituitary glycoprotein hormone α-subunit gene for TR response element (TRE) (nucleotides −74 to −38) and the preparation of nuclear extract have been described (3, 4).

RESULTS

Structural Analysis of the rTRα-1 Region Between Residues 288–331. A schematic representation of rTRα-1 and the location and primary sequence of residues 288–331 is given in Fig. 1A. Alignment of the homologous region from other TRs shows a high degree of protein sequence conservation. Computer calculations [University of Wisconsin Genetics Computer Group, sequence analysis software package version 6.2 (16)] predict a potential α-helical region(s) flanked by a major turn at each end. An amphipathic α-helix might exist between amino acids 292–308, as shown in a helical wheel projection in Fig. 1B. Leu-292, Ile-299, and Leu-306 would appear on the same face of the helix, together with neighboring residues Val-295, Leu-302, and Phe-309. Jointly, they would create an uninterrupted hydrophobic face spanning...

Abbreviations: T3, 3,5,3'-triiodothyronine; TR, thyroid hormone receptor; LBD, ligand-binding domain; TRAP, TR auxiliary protein(s); CAT, chloramphenicol acetyltransferase; rTRα-1, rat TRα-1; ABCD, avidin–biotin complex DNA-binding; TRE, TR response element.

*To whom reprint requests should be addressed.

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Biochemistry: Spanjaard et al.

**Fig. 1.** (A) Schematic representation of rTRα-1. The DNA- and ligand-binding domains and the location of amino acids (aa) (one-letter code) 288–331 in the receptor are shown. Below is the alignment with other TRα and TRβ sequences. Boldface Ts above the overlaid sequence symbolize turns. Prefixes r, h, c, and X denote rat, human, chicken, and Xenopus, respectively. The sequence in rTRα-1 that might adopt an amphipathic α-helical conformation is shown in boldface; residues that form the hydrophobic face are indicated by asterisks, and residues in the hydrophilic face underlined. rTRα-1, amino acids (aa) 288–331 (9); hTRα-1, aa 287–330 (10); cTRα, aa 290–333 (11); XTRα, gene A and gene B, aa 296–339 (12); rTRβ-1, aa 342–385 (13); hTRβ-1, aa 342–385 (14); cTRβ, aa 250–293 (15); XTRβ, gene A and gene B, aa 218–261 (12). (B) Helical wheel projection of rTRα-1 amino acid sequence 292–309, which might adopt an amphipathic α-helical conformation. Residues Leu-292 (N-terminal start point of the helix), Val-295, Ile-299, Leu-302, Leu-306, and Phe-309 form an uninterrupted hydrophobic face spanning five turns of the helix. Charged residues Asp-297, Glu-301, and Lys-304 constitute the hydrophilic face on the opposite side. (C) Schematic overview of mutations in rTRα-1 between residues 288–331. Residues in turns are underlined; those in the α-helix are in boldface type. Numbers correspond to numbers of respective clones used in text. Double or triple mutations are indicated above the sequence; single mutations are indicated below. For a list of clones with respective mutations, see Table 1.

Five turns of the helix. In contrast, charged residues Asp-297, Glu-301, and Lys-304 are exclusively found on the opposite side. This kind of structure is thought to be involved in protein–protein interactions and could constitute part of the transactivation and/or dimerization interface (17). This hypothesis was tested by creating point mutations in rTRα-1 that would specifically change α-helical properties of the sequence. Fifteen mutants were constructed (Fig. 1C and Table 1) and assayed for T3 binding, transactivation, DNA binding, and heterodimerization with TRAP.

T3 Binding of rTRα-1 and Point Mutants. In vitro-transcribed mRNA of rTRα-1 and point mutants was translated in vitro with [35S]methionine, and specific binding of 125I-labeled T3 was determined. As a control, equal volumes of the same programmed lysate were analyzed by SDS/PAGE. mRNAs were found to be translated with approximately equal efficiency. Densitometric scanning of autoradiograms was used to normalize the data obtained by T3 binding (data not shown). The results are given in Fig. 2. Somewhat unexpectedly, this region appears important for hormone binding. The conservative substitution of valine for Leu-292 in clone 3 almost completely destroys T3-binding activity. Moving down the α-helical axis, this effect becomes gradually more severe for the other two amino acids aligned on the hydrophobic surface; clone 4 retains 65% T3-binding activity, and clone 5 is indistinguishable from wild type. In accordance with this, the independently constructed double mutation in clone 2 shows no appreciable T3 binding. Interestingly, although Leu-306 can be functionally replaced by valine, substitution by a charged residue (arginine) in clone 11, which would disrupt the hydrophobic face, severely impairs T3 binding. Two other mutations were made to test this interpretation. The adjacent valine residues in positions 294 and 295 were separately mutated into negatively charged amino acids (clones 8 and 9, respectively). We envisaged two possibilities: if this region were randomly structured, similar T3-binding activities for both mutants might be expected.

**Fig. 2.** Relative T3 binding of receptor mutants compared with wild-type rTRα-1 (clone 1; set at 100%). T3 binding of clones 2, 7, 10, 11, and 13 is undetectable (<5%). Experiments are done in triplicate (SEM <1% of mean values) with in vitro-translated [35S]methionine-labeled receptors and 0.5 nM 125I-labeled T3, and normalized for receptor quantity (see text). Results represent mean values. Wt, wild-type; other clones are designated in Table 1.
because they are virtually identical. On the other hand, if this region were α-helical, a charge (glutamic acid) in the hydrophilic face should have limited effects (clone 8), but a charge (aspartic acid) in the hydrophobic face (clone 9) should have deleterious consequences. In accordance with our model, the latter is seen.

Another line of evidence for existence of the α-helix was obtained through mutations that disrupt its continuity. As expected, placing proline in the middle of the α-helix in clone 10 abolishes T3 binding. The same Leu-302 was changed into alanine in clone 15 with much less effect. Next, we investigated whether proline substitutions could be used to determine the N- and C-terminal boundaries of the helix from a functional viewpoint because proline substitutions might have limited effects in these positions. Asn-289 and Asn-310 might be located at the N- and C-terminal borders, respectively, and thus form suitable targets for substitution. In addition, asparagine is statistically favored at the N-terminal end of helices (18) and thought to angulate the α-helix between the basic region and leucine zipper in bZip type of transcription factors (19). As shown, clone 12 has almost wild-type affinity for T3, suggesting that the helix might terminate in this position. However, T3 binding in clone 7 is disrupted, perhaps because of distortion of the turn in which the proline residue is presumably placed. Surprisingly, clone 14 has lower, but measurable, T3-binding activity. Kinking the helix in this proximal position may leave enough C-terminal helix intact for T3 binding, whereas disruption of the hydrophobic face, as in clone 9, is more detrimental.

rTR-1 has three amino acid differences in the helix, corresponding to rTRα-1 positions 301, 304, and 308. To test the importance of these variations, a triple mutation was constructed (clone 16) that makes rTRα-1 identical to rat TRβ in this region. As shown in Fig. 2, these mutations have no measurable effect. Finally, two other constructs were made further downstream. Substitution of valine for Leu-311 (clone 6) does not change the T3-binding activity. The second mutation, in clone 13, strongly interferes with T3 binding, showing that the integrity of the whole region is necessary for proper T3 binding.

Transactivation by rTRα-1 and Its Derivatives. To examine the potential role of the region studied here in transactivation, each construct was cotransfected into receptor-deficient COS-7 cells with a reporter plasmid carrying a T3-inducible promoter regulating transcription of the bacterial CAT gene (20). CAT activity was measured with and without T3, and fold induction was calculated (Fig. 3). Mutants with >40% T3 binding compared with wild type are not significantly changed, indicating that the substituted residues are not specifically involved in transactivation. All clones with <5% T3 binding induce significantly less CAT activity than wild type, but it is impossible to determine whether the measured effects are caused by either reduced affinity for T3, an impaired transactivation potential, and/or some other factor. Clones 2, 10, and 13 are completely inactive in both T3 binding and transactivation. However, in the absence of hormone, all constructs can suppress basal transcription of the target gene, suggesting that their structure is still largely intact (data not shown).

Effect of Point Mutations on Heterodimerization with TRAP. Several reports (3-5, 21-23) have shown that TR heterodimerizes with a nuclear factor(s), or TRAP, resulting in greatly increased stability of TR-DNA complexes. We used the ABCD assay to measure TR–TRAP heterodimer formation of the mutants by using nuclear extract derived from 235-1 cells as a source of TRAP activity. This rat pituitary cell line has no detectable TR activity; the results are shown in Fig. 4. In the absence of nuclear extract, 10% of rTRα-1 is bound to the DNA. As expected, when nuclear extract is added, the number of TR–DNA complexes increases >5-fold. Most mutants display basal DNA binding and TR–TRAP heterodimerization characteristics similar to wild type. As has been shown (3), this mechanism is independent of the ability of the receptor to bind T3. For example, clones 2 and 3 do not bind T3, but the mutations have no effect in this assay. Three mutants (clones 10, 11, and 13) show lower basal DNA binding and are severely impaired in heterodimerization. DNA binding of clone 10, in which the helix is kinked, is poorly enhanced by nuclear extract, whereas mutation of the same Leu-302 into alanine (clone 15) results in normal levels. Disruption of the helix that is N-terminal of position 302 (clones 7, 9, and 14) has no significant effect, but when it is C-terminal (clone 11), basal DNA binding is reduced, and heterodimerization is strongly impaired. Clone 5, the control for clone 10, is much less affected. Also, substitution for proline at the Asn-310 helix boundary is fairly well tolerated (clone 12). This result suggests that within this region the integrity of only the C-terminal portion (residues 302-310) of the helix is important for TR–TRAP heterodimer formation, in contrast to hormone binding, which requires the entire helix. The amino acid changes in this region will be the subject of a future report.

![Fig. 3. Transactivation by rTRα-1 and point mutants. Two and one-half micrograms of receptor DNA was cotransfected into COS-7 cells with 5 μg of reporter plasmid pk14CAT, which contains the bacterial CAT gene under transcriptional control of a TRE (20). As an internal control for transfection efficiency, 3 μg of pR59Luc was included in all experiments, and luciferase activity (Luc) of extracts was used to normalize for CAT activity. CAT activity was measured with or without T3 (100 nM), and ratios were determined (fold induction). Results represent the mean of at least four experiments done in duplicate. C, no receptor (pSG5X); Wt, wild type; other clones are designated in Table 1. *p < 0.05.](image)

![Fig. 4. Heterodimerization of rTRα-1 and point mutants with TRAP. Linearized receptor (mutant) DNA was transcribed in vitro, and mRNA was translated in the rabbit reticulocyte lysate system with [35S]methionine. Binding of each protein to the rat glycoprotein α-subunit TRE was tested in the ABCD assay in the absence (NE, □) or presence (+NE, ●) of 40 μg of nuclear extract (NE) derived from 235-1 cells. Data represent mean values of two experimental points. Wt, wild-type; other clones are designated in Table 1.](image)
acid differences between TRα and TRβ are not significant for TR–TRAP interactions (clone 16). The third mutant that is seriously affected in heterodimerization is clone 13. Whether this result is due to a disrupted structure and/or the impairment of a specific interaction of residue Leu-318 with TRAP is unknown.

**DISCUSSION**

Amino acids 288–331 in TRα-1 are important for T3 binding and formation of TR–TRAP heterodimers. We constructed 15 different point mutations in TRα-1 between residues 288 and 331 and measured their effects on receptor function with regard to ligand binding, DNA binding, transactivation, and heterodimerization with TRAP (Table 1). Previously, sequence analysis of the LBD of chicken TRα revealed eight or nine potential α-helical heptad repeats, which were suggested to constitute the dimerization interface of TR (24, 25). Our results are consistent with the existence of an amphipathic α-helix within the second and third heptad repeat. However, in addition to its role in dimerization, the integrity of the entire helix strongly correlates with T3-binding activity, and residues outside the helix are also involved. Clearly, the organization of functional subdomains in the LBD of the TR is extremely complicated. Mutations causing a loss in both hormone binding and TR–TRAP heterodimer formation were found clustered at the C-terminal portion of the putative helix and further downstream. This configuration is reminiscent of that in the estrogen receptor, where a major ( homo) dimerization domain colocalizes with residues involved in estrogen binding (26). We speculate that the α-helix forms a structural part of the T3-binding pocket, possibly through intramolecular peptide–peptide interactions, resembling the coiled–coil interaction found between leucine zippers (27). It is particularly interesting that Leu-292 cannot be functionally replaced by valine. Computer modeling of the C/EBP leucine zipper predicts that valine would block interdigitiation, instead of locking the coils together (28). Because disruption of the turn at the N terminus of the helix has a strong effect on T3 binding (clone 7), loss of a stabilizing interaction near the turn, as in clone 3, may then have the same result. This result would also explain why the effect of valine substitutions in clones 4 and 5 becomes gradually less dramatic as the distance from the turn increases. At present, it is unclear how, mechanistically, this region interacts with TRAP, but it may be a combination of structural component(s) like the α-helix and individual interactions between residues in TR and TRAP.

rTRα-1 and rTRβ-1 differ by three amino acids in the helical region, but our data indicate that these variations are not important. Therefore, an explanation for the defect in one pedigree with generalized resistance to T3 due to a Gly-345 (corresponding to Gly-291 in rTRα-1) to arginine substitution in hTRα-1 (29) may be that the defect in T3 binding is caused by a disrupted turn (Lys-Asn-Gly-Gly) at the N-terminal end of the helix, or interference with the action of the adjacent leucine residue, as described above. This mutant could still heterodimerize with TRAP. Unfortunately, the role of TR– TRAP heterodimers in transactivation cannot be evaluated because the mutants that exhibit decreased TR–TRAP for-

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Mutations are designated as follows: residue 306αTR-substitution. Clone 16 is a triple mutation E301, K304, A306αTR–D, M, S, which is identical to rat TRβ in the helix. Relative activity compared with wild type (clone 1) is as follows: +++, 75–100%; +++, 50–75%; ++, 25–50%; +, 5–25%; +/−, <5%.

![Fig. 5. The region in rTRα-1 involved in heterodimerization with TRAP, aligned with homologous regions in some other (putative) receptors (adapted from ref. 34). Gaps have been introduced for maximum fit. Conserved amino acids are boxed. Critical amino acids for rTRα–1–TRAP heterodimer formation, as described in text, are indicated by vertical arrows. The derived consensus (Cons.) sequence is given below: X*, hydrophobic amino acid residue; * denotes a critical amino acid.](image-url)
nformation are also compromised in TRβ binding, but clearly the mere ability to heterodimerize with TRAP is insufficient to generate a transcriptionally active receptor. The observation that the double-defective mutants have relatively lower basal DNA-binding levels may be explained by the presence of some TRα-like activity in reticulocyte lysate, resulting in a higher base line for receptors that can form heterodimers.

**Topology and Conservation of Dimerization Subdomains.**

The TR can form homodimers (3, 23, 30) and heterodimers with nuclear hormone receptors (31) and/or with unidentified nuclear protein(s) (3–5, 21–23) through the LBD. The question arises how many subdomains are involved and whether the same regions/residues are used in all interactions. In the estrogen receptor, two conserved homodimerization domains have been identified: (i) one that coincides with the nuclear localization signal (32), and (ii) a stretch of 22 amino acids located in the C terminus (26, 33) that can be aligned with the C-terminal heptad repeat in TR (26). With respect to heterodimerization domains, apart from the one reported here, there is evidence for the involvement of two other regions in TR: (i) within the nuclear localization signal, and (ii) one identified in rTRβ-1 in the conserved region 286–305 (rTRβ-1 amino acid numbering) (3, 5). Thus, with regard to the primary sequence, four different noncontiguous regions may contribute to the homo- and/or heterodimerization interface.

Comparison of the amino acid sequence of the human homolog of the chicken ovalbumin upstream promoter transcription factor with the sequences of other members of the receptor superfamily revealed that two regions (II and III) in the LBD are relatively well-conserved (34). These regions map to the heterodimerization domains in the TR identified by us (region III) and others (region II (5)). Fig. 5 shows the alignment of amino acids 302–331 of rTRα-1 with the homologous regions of other members of the superfamily; introduction of gaps in the alignment permits derivation of an extensive consensus sequence. The alignment clearly shows that the three amino acids we identified as important for TR–TRAP heterodimer formation are highly conserved. Many nuclear hormone receptors have now been found to interact with different nuclear protein(s) (ref. 3 and the references therein). It will be important to determine which conserved residues play roles and whether the differences in sequence contribute to the specificity of binding to different nuclear protein(s).

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