Thyroid Hormone Receptor Dimerization Is Required for Dominant Negative Inhibition by Mutations That Cause Thyroid Hormone Resistance*

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(Received for publication, November 17, 1992, and in revised form, February 23, 1993)

The syndrome of thyroid hormone resistance (THR) is caused by multiple distinct mutations of the ligand-binding domain of the thyroid hormone β receptor. Although the mutant receptors are transcriptionally inactive, they inhibit normal receptor function in a dominant negative manner to cause hormone resistance. Because most of the naturally occurring mutations are clustered within two areas that lie on either side of a putative dimerization region, we hypothesized that receptor dimerization was important for dominant negative inhibition. In gel mobility shift assays, two THR mutants (G345R and P453H) formed homodimers as well as heterodimers with the retinoic acid X receptor α. In contrast, an artificial mutation (L428R) in one of the hydrophobic heptad repeats of the putative receptor dimerization domain impaired heterodimerization with retinoic acid X receptor α without altering the formation of homodimers. Double mutants containing either of the THR mutations along with the dimerization mutation formed homodimers but not heterodimers, reflecting the properties of the dimerization mutant alone. In transient expression assays using positively (TRETKLuc) or negatively (TSHαLuc) regulated reporter genes, the dominant negative activity of the THR mutants was eliminated by the addition of the dimerization mutation. These results support a mechanism for dominant negative activity by THR mutants in which functionally inactive heterodimers bind to DNA to inhibit access by normal receptors.

As implied by its name, the syndrome of thyroid hormone resistance (THR) is characterized by target organ resistance to thyroid hormone. One of the characteristic features of the disorder is an alteration in circulating thyroid hormone levels. Free thyroid hormone levels are elevated in the setting of inappropriately normal or slightly increased levels of thyroid stimulating hormone (TSH) (1). This pattern of hormones reflects a compensated state in which diminished feedback inhibition of the hypothalamic-pituitary axis by thyroid hormone results in TSH stimulation of the thyroid gland to produce a higher steady state level of thyroid hormones. Thyroid hormone resistance occurs to a variable degree in other target organs, but is more difficult to quantify than the alterations in the thyroid axis (2). Additional features of the syndrome can include goiter, mental retardation, attention deficit disorders, language abnormalities, tachycardia, and delayed skeletal maturation (3). In almost all cases, the syndrome is inherited in an autosomal dominant manner (3, 4).

Thyroid hormone action is mediated by binding to nuclear receptors that activate or repress the transcription of specific target genes. There are two thyroid hormone receptor genes, α and β, as well as splicing variants of each, resulting in a variety of receptor isofoms (5). There is now extensive evidence that THR is caused by mutations in the β form of the thyroid hormone receptor (TRβ) (3, 4). More than 20 distinct mutations have now been described in the ligand-binding domain of TRβ. In most, but not all cases, the mutations greatly decrease thyroid hormone binding affinity (3, 4, 6). In all cases that have been studied, the mutant receptors lack transcriptional activation or repression in transient expression assays (7-10). Thus, the mutations appear to inactivate the receptor either by reducing thyroid hormone binding and/or altering critical domains involved in transcriptional control.

Although it is plausible that a mutation in one of the β receptor alleles could result in the partial resistance that is seen in THR, evidence derived from an unusual kindred with an autosomal recessive form of THR suggests that inactivation of a single allele is not sufficient to cause the syndrome (11). In this family, there is a large deletion that includes both DNA-binding and ligand-binding domains of the β receptor (12). Although individuals homozygous for this β receptor deletion exhibited hormone resistance, the heterozygotes were normal. This observation is consistent with the concept that the more common autosomal dominant form of THR is caused by mutant receptor inhibition of normal receptor function in a dominant negative manner (7, 8). Dominant negative activity of THR mutants has now been documented in transient expression assays, and it occurs with respect to genes that are stimulated or repressed by thyroid hormone (7, 8, 10).

Three potential mechanisms have been proposed to explain the dominant negative activity of receptor mutants: 1) competition of the mutant receptor and normal receptor for the interactions with target DNA sequences; 2) functionally inactive heterodimer formation between normal and mutant receptors; and 3) titration of limiting transcriptional cofactors (8). In previous studies, we provided evidence that competition at the target DNA-binding site is the major mechanism for the dominant negative activity of THR mutants (13). This conclusion was based upon the observation that addition of DNA-binding mutations into the THR mutants abolished
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their dominant negative activities. Recently, thyroid hormone receptors have been shown to bind to DNA as homodimers (14–16) or as heterodimers with accessory proteins such as members of retinoid X receptor (RXR) family (17–21). Dimerization appears to be important for stabilizing receptor-DNA complexes (15, 22) and could therefore play an important role in receptor action.

The dimerization domain of the thyroid hormone receptor has been proposed to consist of a series of repeated hydrophobic motifs in the carboxyl terminus that overlap with the ligand-binding domain (23). It is notable that the receptor mutations that occur in THR are clustered in two discrete domains (centered at amino acids 345 and 453) that are separated by about 100 amino acids of the putative dimerization region (24, 25). This finding raises the possibility that dimerization may be required for the mutant receptors to exert dominant negative activity. In this report, we examined the role of thyroid hormone receptor homodimerization and heterodimerization in the dominant negative activity of THR mutants.

MATERIALS AND METHODS

Plasmid Construction and Receptor Mutagenesis—The TRETK luciferase gene (Luc) contains two copies of a palindromic thyroid hormone-responsive element (TRE) upstream of the thymidine kinase promoter (26) in the PA luciferase vector (27). The TRETK Luc contains 846 base pairs of 5'-flanking sequence and 44 base pairs of the promoter (26) in the PA, luciferase vector (27). TSHα Luc was constructed by the exchange of DrAl-BsmI fragment of the receptor cDNA.

The mutant human TSH α cDNAs were prepared by site-directed mutagenesis and verified by DNA sequencing (8, 13). Mutant and wild type receptor cDNAs were subcloned into a Rous sarcoma virus (RSV) driven expression vector (provided by Dr. H. H. Samuels, New York University Medical Center) (29) and the human TSHα subunit gene (30) (provided by Dr. R. M. Evans, University Medical Center) (29) and the pGEM7 plasmid (Promega, Madison, WI). The double mutations (G345R/L428R, P453H/L428R) were constructed by the exchange of DraIII-BsmI fragment of the receptor cDNA.

T3 Binding Assays—T3 binding assays were performed as described previously (13) using a filter binding method. Receptor affinity constants ($K_d$) were calculated using Scatchard plot analyses. The results of at least three individual experiments were used to determine mean ± S.D.

The receptor cDNAs were transcribed in vitro and translated using the TNT-coupled reticulocyte lysate system according to the protocol of the supplier (Promega, Madison, WI). The programmed lysates (3–5 μl) were incubated in a reaction mixture (20 μl) consisting of 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 50 μg/ml poly(dI-dC), and 20 nM $T_3$, at room temperature for 10 min. For studies of dimerization, 0.5 μl of in vitro transcribed, translated hRXRa (30) (provided by Dr. R. M. Evans, Salk Institute) was coincubated with the thyroid receptor. 32P-Labeled oligonucleotides (50 fmol, 50,000 counts/min) were added to this reaction and incubated at room temperature for 20 min. For the studies of the antibody supershift, a specific antibody (provided by Dr. L. J. DeGroot, University of Chicago) was added at 1:100 dilution and incubated further for 1 h on ice. The protein-DNA complexes were analyzed by electrophoresis through a 5% polyacrylamide gel with 0.5 × Tris-borate, EDTA buffer (TBE; 0.045 M Tris-borate, 0.001 M EDTA) and 2.5% glycerol.

Cell Culture and Transient Expression Assays—JEG-3 cells were grown in Optimem (BRL–GIBCO) containing 2% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were transfected 18 h prior to transfection and plated into medium containing 2% fetal bovine serum depleted of $T_3$, with treatment activated charcoal. Triplicate plates of cells were transfected with 5 μg of reporter plasmid (TRETKLuc or TSHα Luc) together with 0.2–2 μg of wild type or mutant receptor expression vector with addition of RSV CAT plasmid (31) as necessary to maintain the total amount of DNA constant. Following a 16-h exposure to a calcium phosphate-DNA precipitate, fresh serum-stripped media was added with or without 5 nM $T_3$. After an additional 48 h, the cells were harvested, and luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) (32).

RESULTS

Structure of Receptor Mutants and Analyses of T3-Binding—A Gly to Arg substitution at amino acid 345 (G345R) (33) and a Pro to His substitution at amino acid 453 (P453H) (34) have been reported in separate kindreds with THR. An artificial mutation was created in the ninth heptad repeat of the putative dimerization domain (23). The hydrophobic residue, Leu428 was substituted with a positively charged amino acid, Arg (L428R). Scatchard analyses were used to determine the $T_3$ binding affinities of the in vitro translated products of each of the receptor mutants. As described previously, the P453H mutant exhibited about 10-fold reduced affinity (2.73 ± 1.5 × 10⁶ M⁻¹) (13). The affinities of G345R and L428R mutants were below detection (normal, 3.09 ± 2.9 × 10⁶ M⁻¹).

Analyses of Mutant Receptor Binding to DNA—Gel mobility shift assays were used to analyze mutant receptor binding to a palindromic (Pal) TRE (26) and to an inverted palindromic (Lap) TRE (35, 36) (Fig. 1). In vitro translated hRXRa forms two protein-DNA complexes (Fig. 1, arrow 1 and 2) with the Pal and Lap TREs. When bound to TRE-

![Fig. 1. Gel mobility shift assays of in vitro translated α and β wild type thyroid hormone receptors using the palindromic and inverted palindromic TREs. In vitro translated α and β wild type receptors were preincubated with or without hRXRa. The protein-DNA complexes bound to the 32P-labeled palindromic (Pal) or inverted palindromic (Lap) TREs were analyzed by non-denaturing gel electrophoresis. The receptor-DNA complexes are indicated as arrows (1, monomer; 2, homodimer; 3, heterodimer).](image-url)
Pal, the faster migrating complex (band 1) was predominant, whereas bands 1 and 2 were of similar intensity when bound to TRE-Lap. Coincubation of TRα with RXRa formed a much slower migrating band (Fig. 1, arrow 3). Using a different DNA sequence that contains a single TRE half-site, only band 1 was seen with TRα (data not shown). Based upon these results and similar published findings (14, 21, 36), the protein-DNA complexes are concluded to represent monomer (band 1), homodimer (band 2), and heterodimer (band 3). In vitro translated TRβ predominantly formed homodimer (band 2), which was very faint with Pal, but prominent with Lap. The addition of RXRa formed a strong heterodimer complex with both TREs. The receptor-DNA complexes with TRβ migrated more slowly than those with TRα1, likely reflecting the larger size of TRβ.

The binding properties of the mutant TRβ were also studied using the palindromic TRE sequence (TRE-Pal) (Fig. 2). The in vitro translated products of β wild type receptor formed a faint receptor-DNA complex with the TRE-Pal, consistent with homodimer (Fig. 2, arrowhead). The addition of TRβ-specific antibody resulted in a supershift of TRβ-containing complexes, forming two slowly migrating bands at the top of the gel. Addition of TRβ together with in vitro translated RXRa resulted in prominent complex that migrated more slowly than the receptor homodimer, consistent with the formation of a TRβ-RXRa heterodimer (Fig. 2, arrow). The binding of the G345R and P453H mutant receptors to the palindromic TRE was similar to the wild type receptor in the absence or presence of RXRa. In the absence of RXRa, binding by the L428R mutant was similar to wild type receptor and addition of β-specific antibody caused a supershift. However, the L428R mutant formed very little heterodimeric complex with RXRa. The double mutants containing the THR mutations and the dimerization mutant (G345R/L428R, P453H/L428R) displayed binding activity that was similar to that of the L428R mutant alone. Addition of 20 nM T3 did not affect the pattern or intensity of receptor complexes bound to TRE-Pal (data not shown).

Receptor binding was also studied using the inverted palindrome of the TRE (TRE-Lap), as this element facilitates receptor binding as a homodimer (Fig. 3). The wild type receptor readily formed a homodimeric complex with TRE-Lap (Fig. 3, arrowhead). Addition of RXRa resulted in formation of a heterodimer complex (Fig. 3, arrow) with a mobility that was indistinguishable from that bound to the TRE-Pal (Fig. 3, right lanes). Addition of 20 nM T3 decreased the amount of homodimeric binding to the TRE-Lap, but it did not change the amount of heterodimer complex (36). The two THR mutants also formed homodimer and heterodimer complexes. However, T3 did not alter homodimer binding substantially, consistent with the reduced affinities of these mutants for thyroid hormone. The L428R mutant and the double mutants formed homodimeric complexes that were similar to wild type receptor, but the amount of heterodimeric complex with RXRa was greatly reduced. T3 did not affect the protein-DNA complexes of these mutants. These results indicate that the THR mutants bind to TRE-Pal or TRE-Lap as homodimers and as heterodimers with RXRa. The L428R mutant and the double mutants (G345R/L428R, P453H/L428R) also bind to these DNA sequences as homodimers, but fail to effectively form the heterodimeric complex. Thus, the L428R mutation appears to selectively alter receptor heterodimerization.

**Functional Properties and Dominant Negative Activity of Mutant Receptors in Transient Expression Assays**—To assess the transcriptional activity of the wild type and mutant receptors, each receptor DNA was transfected into JEG-3 choriocarcinoma cells along with a positively (TRETKLuc) or negatively (TSHaLuc) regulated reporter gene. Receptor activity was analyzed by measuring luciferase activity after treatment with 5 nM T3 (Fig. 4). In the absence of transfected receptor, TRETKLuc activity was stimulated to 1.3-fold and TSHaLuc was repressed to 74% of basal activity by treatment with T3. These effects of T3 likely reflect the presence of a small amount of endogenous thyroid hormone receptor in JEG-3 cells. Transfection of wild type TRα conferred a marked increase in T3-mediated activation and repression of the reporter genes. TRETKLuc activity was stimulated by 24.7-fold and TSHaLuc was repressed to 15%. As shown previously (8), the G345R mutant did not mediate activation of TRETKLuc or repression of TSHaLuc (0.4-fold and 76%, respectively). The P453H mutant exhibited partial responsiveness (7.1-fold in TRETkLuc and repression of TSHaLuc to 40%), consistent with its partial binding to T3 (8). The mutation in the dimerization region (L428R) and the double mutations with THR mutants (G345R/L428R, P453H/L428R) did not activate TRETkLuc or repress TSHaLuc.

Having established the functional capabilities of the individual receptor mutants, their dominant negative activities were determined in the presence of wild type receptor (Fig. 5). Although the THR mutants partially block the activity of wild type receptor at a 1:1 ratio (data not shown), a 1:10 ratio of wild type to mutant receptors was used to more clearly illustrate the dominant negative activity of the mutant receptors. T3-dependent activation of TRETKLuc by wild type receptor (25-fold) was inhibited by cotransfection with G345R and P453H mutants (1- and 3-fold, respectively). In contrast, cotransfection of the dimerization mutant (L428R) did not inhibit wild type receptor function (18-fold), indicating that it is not a dominant negative mutant receptor. Insertion of
FIG. 3. Gel mobility shift assays of in vitro translated β wild type and mutant receptors using the TRE-Lap. DNA binding studies using the inverted palindrome (TRE-Lap) were performed in the absence or presence of RXRα with or without 20 nM T3. The various reaction components are indicated at the top of the gel. The mobility of receptor complex bound to the TRE-Lap sequence was compared to that bound to the palindromic TRE (Pal) (far right). Receptor-DNA complexes are denoted as: heterodimer (arrow), homodimer (arrowhead).

FIG. 4. Functional properties of β wild type and mutant receptors. Two-hundred ng of β wild type or mutant receptor DNA driven by the RSV promoter was transfected into JEG-3 cells with 5 μg of TRETK Luc or TSHα Luc. Cells were incubated with the absence (black bar) or presence (hatched bar) of 5 nM T3. After 48 h of incubation, luciferase activity was measured and is indicated as arbitrary light units.

The dimerization mutation into the THR mutants (G345R/L428R and P453H/L428R) prevented dominant negative inhibition of the THR mutants (22- and 20-fold, respectively). Similarly, repression of TSHα Luc by the wild type receptor (15%) was blocked by cotransfection of G345R (67%) and to a lesser degree by P453H (42%). The L428R mutant and the two double mutants (G345R/L428R and P453H/L428R) did not inhibit wild type receptor function (23, 18, and 11%, respectively). These results suggest that the dimerization region is critical for the dominant negative activity of the THR mutants for positive and negatively regulated reporter genes.

DISCUSSION

The regulation of thyroid hormone action through its nuclear receptors is much more complex than predicted from physiologic studies that occurred before the receptors were cloned. In addition to the presence of multiple thyroid hormone receptor genes and protein isoforms (5), it has been demonstrated recently that the thyroid receptors can form homodimers as well as heterodimers with a variety of cellular proteins referred to thyroid hormone receptor auxiliary proteins (TRAPs) (15, 17-19, 37-39). There is also a high degree of variability in the primary sequence and arrangement of thyroid hormone response elements (TREs) (40). We have been interested in studying the naturally occurring mutations in the TRβ gene as a probe of thyroid hormone action. The most striking feature of these mutations is their ability to function in a dominant negative manner to block the activity of wild type thyroid hormone receptors (7, 8). The dominant negative activity of the mutant β receptors is manifest with respect to wild type TRα and TRβ proteins. In addition, the mutant receptors block thyroid hormone regulation of positively and negatively regulated genes (8).

Because a large number of mutations that cause THR have now been described, it is possible to begin to derive structure-function implications based upon the locations of the mutations (Fig. 6). In contrast to mutations in the androgen (41) and vitamin D (42) receptors, it is notable that none of the TRβ mutations that cause THR occur in the DNA-binding domain of the receptor. This observation likely reflects a requirement for DNA binding for the dominant negative effect of the mutant thyroid hormone receptors. Previous experiments demonstrating that addition of a DNA-binding domain mutation to the THR mutants are consistent with this hypothesis (13). Although the naturally occurring THR mutations appear to be restricted to the ligand-binding domain of the receptor, there are several reasons to suggest that alteration of thyroid hormone binding is not the only feature common to these mutations. First, some THR mutations have little effect on thyroid hormone binding (24). Second, thyroid hormone binding is eliminated by mutations in a number of regions in the carboxyl terminus other than the domain affected by naturally occurring THR mutations.2 Third, inspection of the locations of the THR mutations suggests that there is a predisposition for two domains centered around amino acids 345 and 453 (Fig. 6) (24, 25). These features suggest that in addition to alterations in thyroid hormone binding, the locations of the THR mutations have been se-

2 T. Nagaya, unpublished data.
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**Fig. 5.** Dominant negative activity of receptor mutants. β wild type receptor (200 ng) and 10-fold excess (2 μg) of each mutant receptor were cotransfected into JEG-3 cells with each of the Luc reporter genes to determine their inhibitory activities in the presence of 5 nM T3. Fold stimulation of TRETKLuc by T3 or percent relative Luc activity (+T3) are shown.

**Fig. 6.** Schematic summary of mutations that cause thyroid hormone resistance. The thyroid hormone receptor β is illustrated schematically, including central DNA binding (hatched) and carboxyl-terminal ligand binding (stippled) domains. The locations of hydrophobic repeats in the putative dimerization domain are indicated by arrows (23). The ligand-binding domain is expanded to allow illustration of the sites of mutations (6, 9, 47, 48). Mutations are shown in single letter code. Note that in some locations (e.g. 345, 453), more than one type of mutation has occurred. In addition, certain mutations have occurred in several apparently unrelated kindreds (not shown). Deletions and frameshift mutations are shown as Δ and fs, respectively.

lected on the basis of some other aspect of receptor function that is required for dominant negative activity.

The observation that the THR mutations are located on either side of a putative dimerization domain leads to an intriguing hypothesis that mutations that block dimerization would not be capable of dominant negative activity and therefore would not result in a dominant form of THR. The dimerization domain of the thyroid receptor has not been characterized in detail. However, it has been proposed that a series of heptad repeats between amino acids 334 and 428 create a hydrophobic interface that is important for protein-protein interactions (23). The importance of the putative dimerization region was first demonstrated in transient expression assays in which a series of carboxyl-terminal fragments containing this region were shown to inhibit thyroid receptor-mediated transactivation (43). Because these carboxyl-terminal receptor fragments did not include the DNA-binding domain of the receptor, their inhibitory activity was proposed to occur on the basis of forming functionally inactive dimers. Subsequent experiments using gel mobility shift assays have confirmed the ability of the dimerization domain fragments to block the formation of homodimers (15, 44).

Although the dimerization interface appears to involve an extensive region of the carboxyl terminus, the role of the last (the 9th) heptad repeat has been documented most thoroughly (18, 20, 21). For example, deletion of this region prevents formation of homodimers and heterodimers. In addition, the TRα2 splicing variant, which disrupts the 9th heptad repeat, does not form heterodimers effectively (45). The presence of a proline for serine substitution in the 9th heptad region of truncated mutant (entire deletion of amino terminus and DNA binding region) of c-erbA has also been shown to impair dominant negative activity by reducing dimerization (46). For these reasons, we created a mutation in one of the hydrophobic residues (Leu4) of the 9th heptad repeat to test the requirement for dimerization for dominant negative activity of the THR mutants. Two THR mutants were selected to represent examples of the mutation clusters that are proximal (G345R) or distal (P453H) to the dimerization domain.

In the studies of mutant receptor binding to DNA, both types of THR mutants (G345R, P453H) bound to TRE sequences as homodimers or as heterodimers with RXRα (Figs. 2 and 3). The L428R mutant bound to TREs as a homodimer, but failed to form the strong heterodimer complex with RXRα. This finding was unexpected and suggests that the
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Acknowledgments—We are grateful to Dr. R. M. Evans for providing the human thyroid hormone β receptor and RXRα cDNAs, to Dr. H. H. Samuels for the RSV expression vector, and to Drs. E. Macchia and L. J. DeGroot for the TRβ-specific antibody.