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Research Article

Generalized resistance to thyroid hormone (GRTH) is a syndrome of hyposensitivity to triiodothyronine (T3) that displays autosomal dominant inheritance. The genetic defect commonly lies in the ligand-binding domain of one of the TR beta alleles. Since there are two major thyroid hormone receptor (TR) isoforms, TR alpha and TR beta, it is not known how the mutant receptor mediates a dominant negative effect. Previously, we showed that T3 caused dissociation of TR homodimers and TR alpha/TR beta dimers from several thyroid hormone response elements (TREs). Hence, we used the electrophoretic mobility shift assay to compare the effect of T3 on the DNA binding of mutant TR beta-1 (Mf-1) from a kindred with GRTH with normal TR beta. Mf-1 bound better as a homodimer than TR beta, but dissociated from DNA only at high T3 concentrations. Both receptors heterodimerized with nuclear auxiliary proteins. They also dimerized with TR alpha and with each other. Surprisingly, T3 disrupted the DNA binding of the Mf-1/TR isoform dimers. Thus, mechanisms for the dominant negative effect by mutant TRs likely involve either increased binding to TREs by mutant homodimers that cannot bind T3 (hence cannot dissociate from DNA) and/or the formation of inactive mutant TR/nuclear protein heterodimers.

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New Insights on the Mechanism(s) of the Dominant Negative Effect of Mutant Thyroid Hormone Receptor in Generalized Resistance to Thyroid Hormone

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Abstract

Generalized resistance to thyroid hormone (GRTH) is a syndrome of hyposensitivity to triiodothyronine (T_3) that displays autosomal dominant inheritance. The genetic defect commonly lies in the ligand-binding domain of one of the $TR\beta$ alleles. Since there are two major thyroid hormone receptor (TR) isoforms, $TR\alpha$ and $TR\beta$, it is not known how the mutant receptor mediates a dominant negative effect. Previously, we showed that T_3 caused dissociation of TR homodimers and $TR\alpha/TR\beta$ dimers from several thyroid hormone response elements (TREs). Hence, we used the electrophoretic mobility shift assay to compare the effect of T_3 on the DNA binding of mutant $TR\beta$ -1 (Mf-1) from a kindred with GRTH with normal $TR\beta$. Mf-1 bound better as a homodimer than $TR\beta$, but dissociated from DNA only at high T_3 concentrations. Both receptors heterodimerized with nuclear auxiliary proteins. They also dimerized with $TR\alpha$ and with each other. Surprisingly, T_3 disrupted the DNA binding of the Mf-1/ TR isoform dimers. Thus, mechanisms for the dominant negative effect by mutant TRs likely involve either increased binding to TREs by mutant homodimers that cannot bind T_3 (hence cannot dissociate from DNA) and/or the formation of inactive mutant TR/nuclear protein heterodimers. (*J. Clin. Invest.* 1992. 90:1825-1831.) Key words: thyroid hormone receptor • thyroid hormone resistance • homodimer • heterodimer • DNA binding

Introduction

Generalized resistance to thyroid hormone (GRTH)¹ is a syndrome characterized by elevated circulating levels of thyroxine (T_4) and triiodothyronine (T_3) with inappropriately normal or increased levels of thyroid-stimulating hormone (1, 2). Pa-

tients with this syndrome have partial refractoriness to the effects of thyroid hormone, which is variable in individual target tissues. Furthermore, the clinical phenotypes are different among affected families (2, 3). The pattern of inheritance of GRTH is autosomal dominant in the majority of cases (2, 3), although the occurrence of autosomal recessive transmission has been documented in one family (4).

Although thyroid hormone receptor (TR) mutations had been suspected based on decreased thyroid hormone binding in fibroblasts of affected patients (3, 5), direct demonstration of TR mutations could not be undertaken until cDNAs encoding TRs had been identified. Recently, two different thyroid hormone receptor genes, $hTR\alpha$ and $hTR\beta$, that encode highly homologous DNA-binding proteins that bind T_3 have been identified in humans (6-8). The TRs are members of a large superfamily of receptors, including the estrogen, glucocorticoid, vitamin D, and retinoic acid receptors (9, 10). In common with other members, they have several domains, including a central DNA-binding domain containing two "zinc fingers" and a carboxyl-terminal ligand-binding domain.

Recent studies of subjects with GRTH have identified single amino acid substitutions in the ligand-binding domain of their $TR\beta$ -1 (11-15). In the first two families described, the mutant $TR\beta$ -1 had either glycine 345 replaced by arginine (Mf-1) or proline 453 replaced by histidine (11, 12). Mutations in all families with GRTH so far characterized cluster near those described in these two families (15, 16). Affected members of these families have one normal and one abnormal $TR\beta$ allele, consistent with the autosomal dominant mode of inheritance predicted by pedigree analysis. In vitro transcription and translation of the mutant receptors in some of the families showed either reduced or virtually absent T_3 -binding affinity (13, 16, 17). Transient transfection assays using two of the $TR\beta$ -1 point mutants (including Mf-1) have shown that they not only fail to mediate normal T_3 -regulated transcription but also can block the T_3 -regulated transcription by normal TRs (18, 19). The mechanism of this dominant negative effect of the mutant $TR\beta$ -1 allele on the normal $TR\beta$ and $TR\alpha$ -1 isoforms is open to speculation. It has been hypothesized that the mutant $TR\beta$ -1 can dimerize with normal TRs interfering with their ability to mediate ligand-regulated transcription (18-20). Indeed, it has been recently shown that $TR\beta$ can homodimerize (21-25) and dimerize with other TR isoforms (25) on DNA sequences containing thyroid hormone response elements (TREs) from thyroid hormone-regulated genes.

Several observations have complicated the elucidation of the molecular mechanism for thyroid hormone resistance. First, a nuclear protein(s), designated thyroid hormone receptor auxiliary protein (TRAP), has been shown to heterodimerize with TRs and enhance their binding to TREs (21, 26-29).

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1. Abbreviations used in this paper: EC_{50} , 50% effective concentration; EMSA, electrophoretic mobility shift assay; GRTH, generalized resistance to thyroid hormone; RXR, retinoid X receptor; TR, thyroid hormone receptor; TRAP, thyroid hormone receptor auxiliary protein; TRE, thyroid hormone response element.

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Second, addition of T_3 can cause dissociation of TR α -1 and TR β -1 homodimers, as well as TR α -1/TR β -1 dimers from certain TREs (25). In contrast, TR/TRAP heterodimers remain bound to these TREs in the presence of T_3 , suggesting that the heterodimer, not the homodimer, may be the functionally relevant receptor complex in ligand-mediated transcription. Lastly, Hamada et al. (30) recently cloned a protein, H-2RIIBP, that bound to a MHC class I gene regulatory element and vitellogenin gene A2 estrogen response element. This protein has high sequence homology with retinoid X receptor α (RXR α) and is now designated RXR β (31). These proteins also bind to the β -retinoic acid receptor gene regulatory element (31, 32). Additionally, they heterodimerize with TRs, resulting in enhanced TR binding to TREs and increased levels of T_3 -mediated transcription (31–35). RXRs are members of the same superfamily of receptors as TRs and bind to a putative endogenous ligand, 9-*cis* retinoic acid (36). Recently, T_3 has been shown to have no effect on TR/RXR heterodimer binding to TREs (36a).

To better understand the mechanism whereby mutant TRs of patients with GRTH exert a dominant negative effect, we compared the *in vitro* DNA-binding of a mutant TR β (Mf-1) from kindred Mf with native human TR β -1. In particular, we examined homodimerization, receptor isoform dimerization, and receptor heterodimerization with TRAP and RXR β , using the electrophoretic mobility shift assay (EMSA). Additionally, we examined the effect of T_3 on the binding of these complexes. On the basis of our results, we propose that the mutant TR does not exert its dominant negative effect by direct dimerization with native TR α -1 or TR β -1 from the normal allele, since T_3 induces a normal dissociation of these complexes from DNA. Instead, the occupation of TREs by mutant homodimers that cannot bind T_3 (hence cannot dissociate from DNA) and/or the formation of inactive mutant/TRAP complexes may be the mechanism(s) by which these mutant receptors mediate resistance to thyroid hormone.

Methods

Preparation of *in vitro* translated receptors. Previously described cDNA clones of rat TR α -1 (TR α -1), human TR β -1 (TR β -1), Mf-1, and ΔN in pGEM (kindly provided by Dr. D. S. Darling, Brigham and Women's Hospital, Boston, MA), and mouse RXR β (or H-2RIIBP) in pBS (kindly provided by Dr. K. Ozato, National Cancer Institute, Bethesda, MD) were used in these experiments (6, 28, 30, 37, 38). Each cDNA was linearized with the appropriate restriction endonuclease and used as a template for RNA synthesis with T_7 RNA polymerase. Unlabeled and [35 S]methionine-labeled receptors then were produced from rabbit reticulocyte lysates according to the manufacturer's instructions (Bethesda Research Laboratories, Bethesda, MD). Unprogrammed reticulocyte lysate was also incubated under the same conditions. The amount of translated protein was quantitated by TCA precipitation (26, 39) and by SDS-PAGE analysis of [35 S]methionine-labeled receptors that showed labeled proteins of expected molecular weights.

Preparation of nuclear extracts. Nuclear extract from the rat pituitary lactotropic cell line 235-1 was prepared and stored as previously described (26). These cells do not bind T_3 , and so presumably do not have endogenous TRs. Extracts then were dialyzed against 20 mM Hepes (pH 7.3), 5 mM 2-mercaptoethanol, 50 mM NaCl, 2 mM EGTA, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride and were centrifuged 10,000 *g* for 15 min. Aliquots were stored frozen at -70°C until used in DNA-binding assays.

Design and preparation of labeled DNA probes. Double-stranded oligonucleotides containing TREs from either the chicken lysozyme gene (F2) –2344 to –2326 (40) or direct repeats of two half sites (AGGTCA) separated by a gap of four nucleotides (DR4) (41) in the context of the F2 flanking sequences were used in our experiments. The oligonucleotides were end-labeled with [32 P] γ -ATP by T4 polynucleotide kinase. The labeled probes were then purified on a 9% polyacrylamide gel under nondenaturing conditions. The full-length probe was cut from the gel and extracted in 400 μ l 0.5 M ammonium acetate and 1 mM EDTA at 37°C for 4 h. After microcentrifugation, labeled probe was extracted from the supernatant by ethanol precipitation. The pellet was washed with 70% ethanol, resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and stored at -20°C until used in DNA-binding assays.

DNA-binding assay/EMSA. Unlabeled receptor preparations or unprogrammed reticulocyte lysate (0.5–6.0 μ l) and 10,000 cpm oligonucleotide probe were incubated in 20 μ l of 25 mM Hepes (pH 7.5), 5 mM MgCl $_2$, 4 mM EDTA, 2 mM dithiothreitol, 110 mM NaCl, 5 μ g/ml bovine serum albumin, and 0.8 μ g sheared salmon sperm DNA (Sigma Chemical Co., St. Louis MO) for 30 min at room temperature. Different concentrations of 3,5,3'-L-triiodothyronine (T_3) (Sigma Chemical Co.) were also added to the samples in certain experiments. To study TR/TRAP heterodimerization, 1.0 μ g nuclear extract was added to each sample. To study receptor isoform dimerization or heterodimerization with RXR β , we preincubated samples at 30°C for 10 min before adding oligonucleotide probe. After incubation, samples were then subjected to electrophoresis on 4% polyacrylamide gels in 0.5 \times TBE buffer (45 mM Tris-borate, and 1 mM EDTA) for 75 min at 4°C . Proteins on the gel were fixed by incubation in 7% acetic acid and 10% isopropanol for 15 min at room temperature. The gels were dried under vacuum at 80°C for 1 h and autoradiographed with an intensifying screen. Film exposure to the gels ranged from 12 to 48 h.

Results

We first examined the binding of native human TR β -1 and Mf-1 to a labeled oligonucleotide containing the chick lysozyme gene TRE (F2) by using EMSA. As previously shown with TR β -1 (25), Mf-1 bound almost exclusively as a homodimer on this TRE (Fig. 1 A). Interestingly, Mf-1 bound better than TR β -1 to this TRE since the same amount of *in vitro*-translated receptor (as quantitated by TCA precipitation and SDS-PAGE analyses of ^{35}S -labeled receptor) was added to each DNA-binding reaction. Additionally, the bound Mf-1 homodimer band moved with a slower mobility than the TR β -1 homodimer band (Fig. 1 A). A similar difference in mobility was seen in the faint monomer bands for these receptors on longer exposures of the gel (data not shown). Scatchard analyses of homodimer binding to F2 (Fig. 2) showed that both receptor homodimers had a similar affinity for F2 (TR β -1 $K_d = 0.78$ nM, Mf-1 $K_d = 1.2$ nM); however, total Mf-1 homodimer bound to F2 was threefold greater than TR β -1 for the same amount of *in vitro*-translated receptor added to the DNA-binding reaction (TR β -1 homodimer bound [N_{\max}] = 0.775 fm/reaction, Mf-1 homodimer bound [N_{\max}] = 2.46 fm/reaction). When T_3 was added to the DNA-binding reactions, TR β -1 homodimer binding decreased with an 50% effective concentration (EC_{50}) of $\sim 1.0 \times 10^{-9}$ M, (Fig. 1 A), as reported previously (25). In contrast, T_3 did not decrease Mf-1 homodimer binding to F2 unless much higher concentrations of T_3 were added (EC_{50} of $\sim 1.0 \times 10^{-6}$ M) (Fig. 1 A and B). This diminished T_3 effect on Mf-1 DNA-binding is consistent with the minimal T_3 binding previously reported for *in vitro*-translated Mf-1 (18, 19).

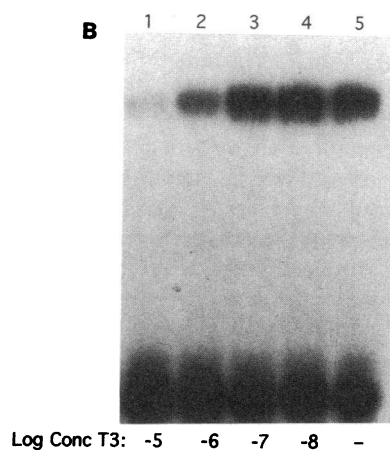
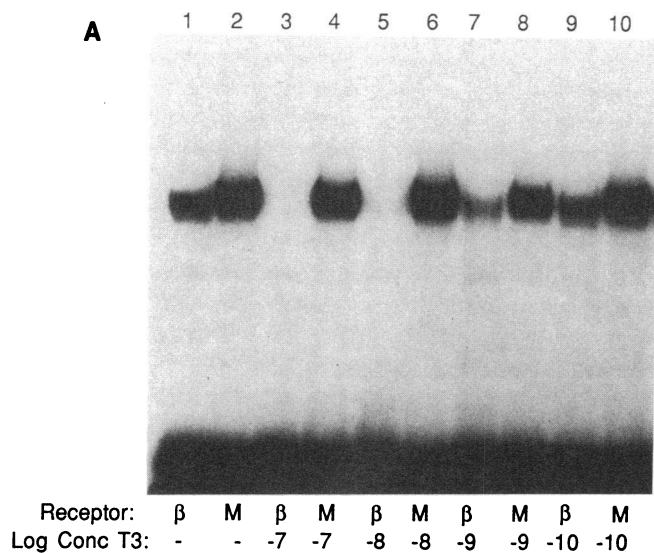


Figure 1. T3 effect on TR β -1 and Mf-1 homodimer binding to F2. Equal concentrations of in vitro translated TR β -1 and Mf-1 and labeled F2 probe were used in the absence or presence of varying concentrations of T3 for analysis by EMSA as described in Methods. Lanes are numbered 1–10 on top of the figure and the additives are indicated at the bottom. β , wild-type TR β -1; M, the mutant TR β (Mf-1).

We next examined whether Mf-1 could form heterodimers with TRAP on two different TREs, F2 and DR4 (a synthetic, positively regulated TRE in which half-sites are oriented as direct repeats and separated by a gap of four nucleotides) (41). Both TR β -1 and Mf-1 formed heterodimers with TRAP on these TREs when incubated with pituitary nuclear extract (Fig. 3, lanes 4, 6, 10, and 12). However, the Mf-1/TRAP heterodimer band migrated slightly slower than the TR β -1/TRAP

heterodimer band. Additionally, the pattern of heterodimer and homodimer binding to the TREs was different for TR β -1 and Mf-1. TR β -1 formed heterodimers preferentially over homodimers on these TREs. As previously reported, when T₃ was added to TR β -1 and nuclear extract, homodimer no longer bound to DNA but heterodimer remained bound to F2. In contrast, both Mf-1 and Mf-1/TRAP heterodimer remained bound to F2 in the presence of 10⁻⁷ M T₃ (data not shown). We also examined whether these receptors could form heterodimers with RXR β , a member of the steroid hormone receptor/TR superfamily that binds to the MHC Class I gene regulatory region and β -retinoic acid receptor gene (28, 29, 35). Both

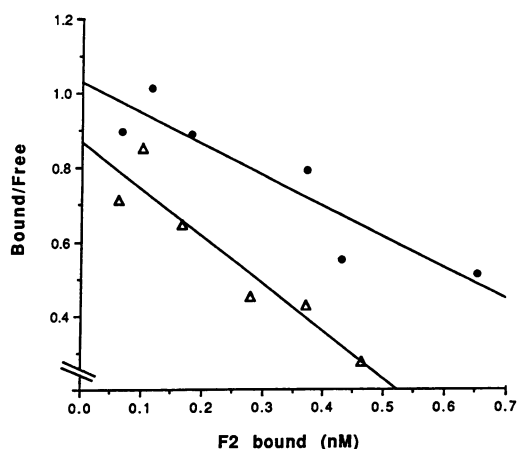


Figure 2. Scatchard analysis of TR β -1 and Mf-1 homodimer binding to F2. Increasing amounts of labeled F2 probe (1,250–40,000 cpm/reaction) were incubated with TR β -1 and Mf-1 and then were analyzed by EMSA. The radioactivity in bands containing bound homodimer and free probe were quantitated by counting the corresponding gel sections. Each point represents the mean of duplicate determinations. (TR β -1 (Δ): $K_d = 0.78$ nM, $N_{max} = 1.39$ fm/reaction, $r = 0.90$; Mf-1 (\bullet): $K_d = 1.20$ nM, $N_{max} = 2.46$ fm/reaction, $r = 0.84$). Note that 1.75-fold more TR β -1 was added so that the amount bound to F2 in the absence of T3 would be equivalent to that of Mf-1. TR β -1 N_{max} normalized for the amount of added receptor = 0.775 fm/reaction. Multiple linear regression analyses of the slopes showed no significant difference between the two K_d s ($P = 0.16$). Similar results were obtained in a second experiment.

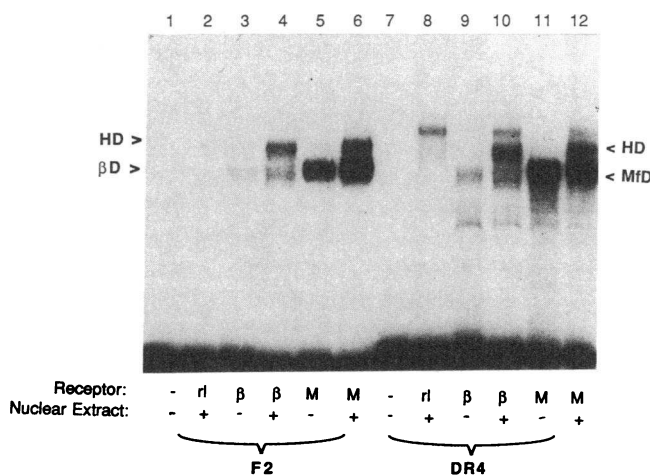


Figure 3. Comparison of TR β -1 and Mf-1 homodimers and receptor/TRAP heterodimer binding to two TREs (F2 and DR4). Equal amounts of TR β -1 and Mf-1 were incubated with 1 μ g of nuclear extract from pituitary 235-1 cells and either F2 or DR4 probe and were analyzed by EMSA. Lanes are numbered 1–12 and the presence (+) or absence (-) of additives are indicated. rl, reticulocyte lysate; β , TR β -1; M, Mf-1; HD, receptor/TRAP heterodimer; β D, TR β -1 homodimer; and MfD, Mf-1 homodimer.

TR β -1 and Mf-1 formed receptor/RXR β heterodimers on F2 and DR4 (Fig. 4, lanes 3, 6, and 9) that were stable after T₃ addition (data not shown). Also, the receptor/TRAP heterodimer band migrated more slowly than the receptor/RXR β heterodimer band, suggesting that TRAP in the pituitary may be different than RXR β (Fig. 4, lanes 3, 4, 6, 7, 9, and 10).

To examine whether the mutant receptor could exert its dominant negative effect via direct interaction with other TR isoforms, we compared the formation of TR α -1/TR β -1 dimers with TR α -1/Mf-1 dimers and the effect of T₃ on DNA-binding by these dimers. We previously showed that incubating TR α -1 and TR β -1 together produced TR α -1/TR β -1 dimers bound to F2 and addition of T₃ decreased DNA-binding of these dimers (25). When TR α -1 and Mf-1 were incubated together, there was loss of TR α -1 homodimer band and formation of a new large band composed of mostly TR α -1/Mf-1 dimers (faster migrating) and a small amount of residual Mf-1 homodimers (slower migrating) (Fig. 5, lane 7). The presence of TR α -1/Mf-1 dimers was confirmed by coincubation with anti-TR α and TR β -1 isoform-specific antibodies (42) in the DNA-binding reaction and observation of appropriate supershifts (data not shown). When 10⁻⁷ M T₃ was added to the DNA-binding reactions, T₃ abolished TR α -1/Mf-1 dimer but not Mf-1 homodimer binding to F2 (Fig. 5, lane 9). Only when 10⁻⁶ M T₃ was added did Mf-1 homodimer dissociate from F2 (Fig. 5, lane 8). These data demonstrate that TR α -1/Mf-1 dimer behaves similarly to TR α -1/TR β -1 dimer, as it is more sensitive to the T₃-mediated decrease in homodimer binding to F2 than the Mf-1 homodimer. Moreover, these data suggest that T₃ only needs to bind to TR α -1 for the TR α -1/Mf-1 dimer to dissociate from F2 since Mf-1 most likely does not bind T₃ at 10⁻⁷ M (18, 19) and therefore does not affect Mf-1 homodimer binding to DNA at this concentration (Fig. 1).

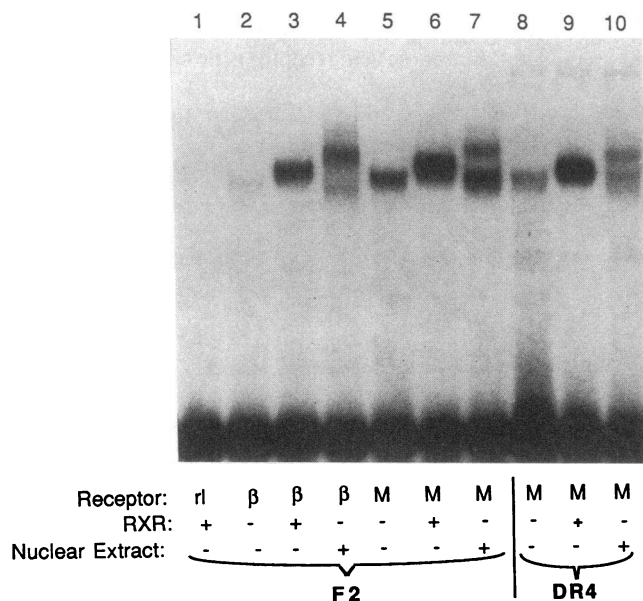


Figure 4. Comparison of TR β -1 and Mf-1 heterodimerization with TRAP and RXR β on two TREs. Equal amounts of TR β -1 and Mf-1 were incubated with 5 μ l in vitro-translated RXR β or 1 μ g of nuclear extract from pituitary 235-1 cells, then reacted with F2 or DR4, and analyzed by EMSA. For abbreviations, see legend to Fig. 3. Note the faster mobility of RXR β /TR than TRAP/TR heterodimers. TRE-bound Mf-1 heterodimers predominated in the presence of TRAP but not RXR β .

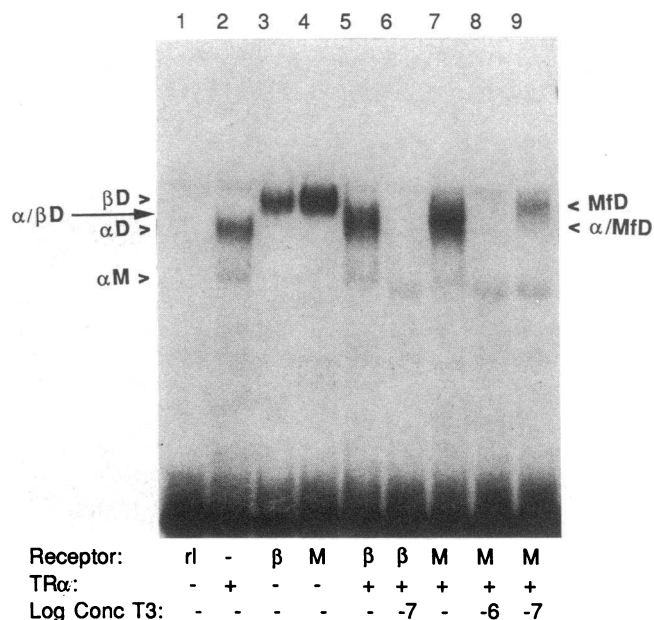


Figure 5. Formation of TR β -1 and Mf-1 dimers with TR α on F2 and the effect of T₃. TR β -1 or Mf-1 (0.5 μ l) was incubated with TR α (6 μ l) in the presence and absence of T₃ and was analyzed by EMSA. Note that in order to form similar amounts of bound TR to F2, 1.75-fold more TR β -1 than Mf-1 was added to the DNA-binding reactions. Samples were analyzed on a 5% polyacrylamide gel and run for 90 min at 4°C to enhance separation of the different receptor bands. The presence (+) or absence (-) of additives is indicated. rl, reticulocyte lysate; β , TR β -1; M, Mf-1; α M, TR α monomer; α D, TR α homodimer; β D, TR β -1 homodimer; MfD, Mf-1 homodimer; α/β D, TR α /TR β -1 dimer; α /Mf-1, TR α /Mf-1 dimer.

We next compared the formation of TR β -1 homodimer with Mf-1/TR β -1 dimer since direct interaction of the mutant receptor with wild-type TR β -1 receptor could account for its dominant negative effect. To demonstrate Mf-1/TR β -1 dimer binding we used a mutant rat TR β -1, Δ N, in which amino acids 71-100 were deleted from the amino-terminal end of the receptor (28). The epitopes for the anti-TR β -1 antibody are contained within this deleted region since the synthetic peptide used to generate the antibody was derived from amino acids 73-93. Therefore, when Δ N was mixed with either Mf-1 or TR β -1, we expected the antibody to supershift Mf-1 and TR β -1 homodimers as well as receptor/ Δ N dimers, but not Δ N homodimers, thereby confirming the formation of any receptor/ Δ N dimers. Δ N bound weakly as a homodimer with similar mobility as TR β -1 and dissociated from F2 after the addition of 10⁻⁷ M T₃. (Fig. 6 A, lanes 4 and 5). When it was mixed with TR β -1, an intense band was observed in the region of the homodimer (Fig. 6 A, lane 6), which likely represented TR β -1 homodimers and new TR β -1/ Δ N dimers as these complexes were supershifted with anti-TR β -1 antibody (Fig. 6 C, lanes 1 and 2). Similar results were seen when Δ N was mixed with Mf-1 (Fig. 6 B and C). When 10⁻⁷ M T₃ was added to the DNA-binding reactions containing TR β -1 and Δ N, all the dimer binding was abolished as expected since both TR β -1 and Δ N homodimers dissociated from F2 at this T₃ concentration (Fig. 6 A, lane 7). However, similar to the case of TR α -1/Mf-1 dimers, 10⁻⁷ M T₃ decreased most of the intensity of the large band representing mainly Mf-1/ Δ N dimers (Fig. 6 B, lanes 7 and 10). The small amount of residual bound complexes observed at this T₃ concentration dissociated from F2 at 10⁻⁵ M

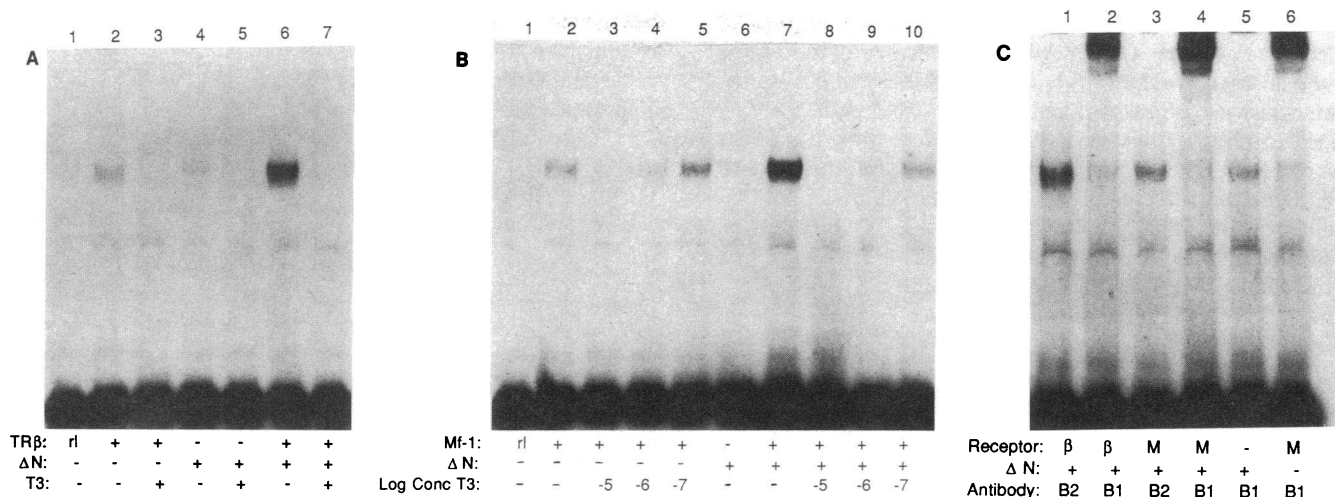


Figure 6. Formation of TRβ-1 and Mf-1 dimers with ΔN on F2 and the effect of T₃. TRβ-1 or Mf-1 (0.5 μl) was incubated with ΔN (6 μl) in the absence and presence of different concentrations of T₃ and was analyzed by EMSA. As in Fig. 5, 1.75-fold more TRβ-1 than MF-1 was added to the DNA-binding reactions. Some samples were incubated with 1:120 diluted rabbit polyclonal antiserum specific for TR isoforms to confirm the formation of dimers. (A) TRβ, ΔN, and TRβ/ΔN dimer binding to F2 in the absence and presence of 10⁻⁷ M T₃. (B) Mf-1 and Mf-1/ΔN binding to F2 in the absence and presence of the indicated concentrations of T₃. (C) Antibody supershift of TRβ-1 and Mf-1 dimers with ΔN. The presence (+) or absence (-) of additives is indicated. rl, reticulocyte lysate; ΔN, wild-type TRβ-1 lacking amino acids 71–100, located on the amino terminus of the DNA binding domain. β, TRβ-1; M, Mf-1; B1, antiserum against the amino terminus of TRβ-1 (amino acids 73–93). B2, antiserum against the amino terminus of TRβ-2, which is different than TRβ-1, used here as control.

T₃ and presumably represented a small amount of DNA-bound Mf-1 homodimers. These results suggest that for Mf-1/ΔN dimer only ΔN needs to bind T₃ for this complex to dissociate from DNA.

Lastly, we mixed equal concentrations of both TRβ-1 and Mf-1 and determined the amount of receptor bound to F2 in the presence or absence of T₃. If Mf-1 homodimerized and dimerized equally well with TRβ-1, the mixture would be composed of ~ 25% Mf-1 homodimers, 50% Mf-1/TRβ-1 dimers, and 25% TRβ-1 homodimers. Furthermore, if Mf-1/TRβ-1 dimer had decreased sensitivity to the T₃-mediated dissociation from DNA as did Mf-1 homodimer, ~ 75% of the receptor mixture would remain bound at 10⁻⁷ M T₃. In contrast, we observed only 33% of the receptor dimer mixture remained bound to F2 at 10⁻⁷ M T₃ (Fig. 7), suggesting that Mf-1/TRβ-1 dimers dissociated from F2. The remaining bound complexes likely represented Mf-1 homodimers for two reasons: Mf-1 homodimers remain bound to F2 at this T₃ concentration, and there are more Mf-1 than TRβ-1 homodimers for the same amount of total receptor (Fig. 2) so they would be expected to represent > 25% of the total receptors. Similar to the studies of Mf-1 dimerization with TRα-1 and ΔN, these results suggest that only one TR (in this case TRβ-1) needs to bind T₃ for the Mf-1/TR dimer to dissociate from DNA.

Discussion

To understand the potential mechanisms by which mutant TRβ-1's exert their dominant negative effect on normal TR function in patients with GRTH, we studied the *in vitro* DNA-binding of Mf-1, which contains a single amino acid substitution (Gly345 to Arg) in the ligand-binding domain. In particular, we compared TRβ-1 and Mf-1 homodimerization, receptor isoform dimerization, and receptor heterodimerization with TRAP and RXRβ, using EMSA. Since it previously had been reported that Mf-1 had minimal T₃ binding, we also examined the effect of T₃ on these receptor complexes.

We found that Mf-1 bound primarily as a homodimer to specific TREs, in a similar manner as TRβ-1. The mobility of both the monomer and dimer bands of Mf-1 were slower on EMSA than for TRβ-1. This change in mobility was probably due to the additional positive charge of the receptor produced by the glycine to arginine substitution. The Mf-1 homodimer bound better than TRβ-1 to two different TREs at the same receptor concentration. Scatchard analyses of receptor homodimer binding to F2 showed that both receptors had similar *K*_ds, but more Mf-1 homodimer bound to the TRE than an equal amount of translated TRβ-1. This suggests that Mf-1 has a larger pool of homodimers capable of binding F2 than TRβ-

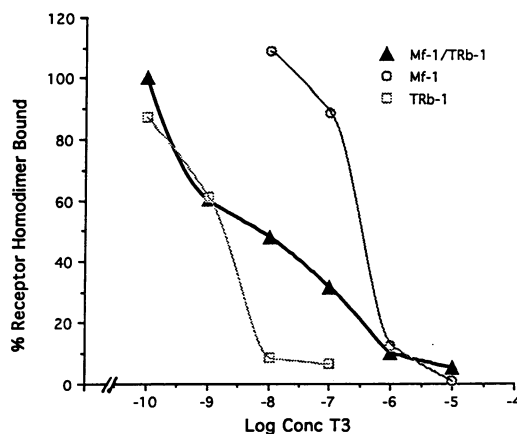


Figure 7. T₃ effect on TRβ-1, Mf-1, and Mf-1/TRβ-1 dimer binding to F2 TRE. Equal amounts of TRβ-1 and Mf-1 receptor were incubated alone or together in the absence or presence of different concentrations of T₃ and were analyzed by EMSA. The densities of dimer bands on the fluorographs were quantitated with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The density of dimer band bound in the absence of T₃ was set as maximal dimer bound (100%). All other bands were normalized as percent dimer bound.

1, perhaps because of greater homodimer stability. Recently it has been shown that TR/TRAP heterodimers form in solution in the absence of DNA (24), so it is possible that TR homodimers also are formed before binding to DNA. When T_3 was added to receptor in the DNA-binding reactions, it induced loss of TR β -1 homodimer binding to F2 (EC_{50} of $\sim 1.0 \times 10^{-9}$ M), as we had reported previously (25). In contrast, Mf-1 remained bound to F2 as a homodimer at 10^{-7} M T_3 and completely dissociated from the TRE only at 10^{-5} M T_3 . These results argue that Mf-1 homodimer may be the major homodimer species bound to TREs in vivo given its greater binding to two TREs of distinctly different orientations (F2 [inverted palindrome] and DR4 [direct repeat]) as well as its markedly diminished ability to dissociate from DNA in the presence of T_3 .

We next examined the ability of Mf-1 to heterodimerize with TRAP on two different TREs, F2 and DR4. We found that, as in the case of TR β -1, Mf-1 heterodimerized with TRAP on both TREs. The mobility of the Mf-1/TRAP band also was slower than the TR β -1/TRAP band. However, when the Mf-1 heterodimer and homodimer binding pattern on these TREs was compared with TR β -1, Mf-1 formed homodimer preferentially to heterodimer, whereas TR β -1 had an opposite tendency. This difference in the dimer binding pattern of the two receptors may be due to the ability of Mf-1 to homodimerize better than TR β -1 on some TREs. As previously reported (25), addition of T_3 to TR β -1 and nuclear extract in DNA-binding reactions abolished homodimer binding to DNA, but not TR β -1/TRAP heterodimer binding. In contrast, addition of T_3 to Mf-1 and nuclear extract did not affect the amount of hetero- or homodimer binding. Recently, it has been shown that RXR β heterodimerizes with TR and enhances TR binding to TREs (30, 31, 34). Moreover, we found that TR β -1/RXR β heterodimers remained bound to TREs in the presence of T_3 , so RXR β may function similarly to TRAP (36a). In our experiments, Mf-1 heterodimerized with RXR β in a similar manner as TR β -1. Also, the receptor/RXR β heterodimer band migrated faster than the receptor/TRAP heterodimer band, and thus it is possible murine RXR β is different than TRAP in the rat pituitary 235-1 cells. Recently the rat RXR β was cloned and found to be virtually identical to its mouse homolog, making it unlikely that species differences in RXR β could account for the different migration pattern of the TR/RXR β and TR/pituitary TRAP heterodimers (34).

We next examined the ability of Mf-1 to form dimers with TR α -1 and TR β -1 and the effect of T_3 on these receptor/receptor dimers. Mf-1 formed dimers with TR α -1 and ΔN , a mutant rat TR β -1 that has an internal deletion in the amino terminus. Using isoform-specific antibodies, we confirmed that TR β -1 and Mf-1 formed dimers with TR α -1 and ΔN . Interestingly, 10^{-7} M T_3 disrupted the DNA-binding of dimers of TR β -1 and Mf-1 that were formed with TR α -1 or ΔN . Since Mf-1 homodimer binding to F2 was abolished only at 10^{-5} M T_3 , this suggests that only one member of the TR α -1/Mf-1 and Mf-1/ ΔN dimers needs to bind T_3 in order for the complex to dissociate. Moreover, since Mf-1/receptor dimer dissociated at the same concentration as the TR α -1/TR β -1 and TR β -1/ ΔN dimers, ligand affects the Mf-1/receptor dimers in a similar manner as TR β -1/receptor dimers. A current model for the dominant negative effect by mutant receptors invokes direct interaction of the mutant receptor with normal receptors, creating a pool of inactive mutant receptor/native receptor dimers that cannot respond normally to T_3 . Our data argue

that this is not the case since the Mf-1/receptor dimers behave similarly to TR β -1/receptor dimers in the presence of T_3 .

Several investigators have reported that unliganded TR suppresses basal transcription by positively regulated TREs (43, 44). Additionally, other investigators have reported that v-erbA, a viral oncogene homolog of TR, can block T_3 -mediated transactivation by TRs, perhaps by binding to TREs (45, 46). We previously have proposed that T_3 -induced dissociation of homodimers from TREs may be necessary to remove repression before ligand-bound heterodimer can regulate transcription (25). If this is the case, it is conceivable that a mutant receptor that binds very poorly to T_3 , when bound as a homodimer to a TRE, may maintain a target gene in a tonically repressed state. Increased expression or stability of the mutant TR, as well as increased binding to some TREs, may further augment this repressive ability of the mutant TR. Another possible mechanism for a mutant receptor to decrease sensitivity to T_3 is based on our previous observation that ligand-bound TR/TRAP heterodimer is the only receptor complex that remains bound to several TREs in the presence of T_3 (25). This, in turn, suggests that the TR/TRAP heterodimer may be the functionally relevant receptor form in T_3 -mediated transcription for some genes (25). Since Mf-1 binds poorly to T_3 , the Mf-1/TRAP heterodimer would not be expected to mediate ligand-regulated transcription by this mechanism. Thus, formation of mutant TR/nuclear protein heterodimers may have a dominant negative effect either by titrating limiting amounts of auxiliary proteins or coactivators (squenching) or by competing with wild-type TR/nuclear protein heterodimers for binding to TREs. In a given cell, alterations in the expression of Mf-1 or the stability of Mf-1/TRAP heterodimers compared with normal TR/TRAP heterodimers could affect the population of abnormal heterodimers and thereby determine the amount of T_3 resistance in the cell.

On the basis of our data, we propose that the dominant negative effect by Mf-1 and the decreased sensitivity to T_3 within the cells of the patients with this mutation are not due to abnormal interactions with TR α -1 and TR β -1 by Mf-1. Instead, they likely occur by one or both of the following mechanisms: repression by mutant homodimers that bind to TREs but do not dissociate from the TREs in the presence of T_3 and/or diminished ligand-regulated transcription by Mf-1/TRAP heterodimers since Mf-1 binds poorly to T_3 . The possibility of multiple regulatory steps that might affect these two mechanisms and differences in impairment of T_3 binding among the different TR β -1 mutations observed in kindreds with GRTH may account for the variability in tissue resistance to T_3 in affected patients as well as the differences in phenotypes among kindreds with this syndrome.

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