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Research Article

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Interaction of Human β1 Thyroid Hormone Receptor and its Mutants with DNA and Retinoid X Receptor β

T_3 Response Element–dependent Dominant Negative Potency

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Abstract

Mutations in the human beta thyroid hormone receptor (h-TRβ) gene are associated with the syndrome of generalized resistance to thyroid hormone. We investigated the interaction of three h-TRβ1 mutants representing different types of functional impairment (kindreds ED, OK, and PV) with different response elements for 3,3′,5-triiodothyronine (T_3) and with retinoid X receptor β (RXRβ). The mutant receptors showed an increased tendency to form homodimers on a palindromic T_3-response element (TREpal), a direct repeat (DR + 4), and an inverted palindrome (TRElap). On TRElap, wild type TR binding was decreased by T_3, while the mutant receptors showed a variably decreased degree of dissociation from TRElap in response to T_3. The extent of dissociation was proportional to their T_3 binding affinities. RXRβ induced the formation of h-TRβ1:RXRβ heterodimers equally well for mutants and the wild type h-TRβ1 on these T_3 response elements. However, the T_3-dependent increase in heterodimerization with RXRβ was absent or reduced for the mutant TRs.

Transient transfection studies indicated that the dominant negative potency was several-fold more pronounced on the TRElap as compared to TREpal or DR + 4. In CV-1 and HeLa cells, transfection of RXRβ could not reverse the dominant negative action. These results demonstrate that the binding of mutant h-TRs to DNA, as well as their dominant negative potency, are TRE dependent. In addition, competition for DNA binding, rather than for limiting amounts of RXRβ, is likely to mediate the dominant negative action. (*J. Clin. Invest.* 1993. 92:1986–1993.) Key words: thyroid hormone receptor • generalized resistance to thyroid hormone • T_3-response element • retinoid X receptor • dominant negative action

Introduction

Mutations in the ligand-binding domain (LBD)[1] of one allele of the human β1 thyroid hormone receptor (h-TRβ1) gene can cause the clinical syndromes of generalized resistance to thyroid hormone (GRTH) (1–3). This disease is characterized by the refractoriness of tissues to the action of thyroid hormone. However, a marked variability exists in the organ resistance within individuals with GRTH (3–5). Intriguingly, the dominant inheritance of the disease is not caused by a lack of active h-TRβ1 protein, since heterozygotes with a deleted h-TRβ1 allele are clinically and biochemically normal (6). It has been hypothesized that the mutant h-TRβ1 inhibits the function of the normal h-TR by a dominant negative mechanism. This was confirmed by in vitro studies demonstrating that h-TRβ1 from patients with mutations in the carboxy-terminal part of the LBD have not only reduced T_3-binding and transcriptional activity, but are also able to inhibit normal h-TRα1 and h-TRβ1 function in transient transfection systems (7–9).

We propose to classify the function of mutant TRs into three different phenomenological types. The type I mutations, represented by kindred ED in this study, have a reduced T_3-binding affinity and transcriptional capacity. High levels of T_3 can completely normalize TR function and reverse the dominant negative potency. The recently described kindred OK represents a type II mutation. Its T_3-binding affinity and transcriptional capacity are reduced, but T_3 can neither restore normal TR activity nor reverse the dominant negative potency (10). This mutation therefore impairs independently the T_3-binding as well as presumably a transactivating domain, although this latter point needs to be mechanistically proven. Kindred PV represents a type III mutation, which has neither T_3-binding nor transcriptional activity, and the dominant negative potency is not reversed by T_3.

Three main mechanisms have been proposed to account for the inhibitory property of the three types of mutant h-TRβ1: (*a*) formation of inactive dimers between mutant and normal h-TRs; (*b*) competition of normal and mutant h-TRs for binding to T_3-response elements; and (*c*) competition for limiting amounts of nuclear auxiliary factors. While the first hypothesis could not be experimentally confirmed, substantial evidence supports a mechanism of competition between mutant and normal receptors for DNA-binding while the role of limiting accessory factors remains equivocal (9, 11). One class of these nuclear TR accessory proteins (TRAP) was recently cloned (retinoid X receptor, subtypes α, β, and γ) and shown to be expressed in a tissue-specific manner. RXRs are capable of homodimerizing, as well as of heterodimerizing with TRs, thereby modifying their DNA-binding and transcriptional capacity several-fold (12–21). The natural ligand of RXR was recently shown to be an isomer of all-*trans*-retinoic acid, 9-*cis*-retinoic acid (22, 23).

The present study was designed to compare the interaction of various mutants and normal h-TRβ1 with different positive TREs in the presence and absence of RXRβ. Specifically, we

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1. *Abbreviations used in this paper:* ABCD, avidin-biotin complex DNA binding; GRTH, generalized resistance to thyroid hormone; h-TR, human T_3 receptor; LBD, ligand-binding domain; rGH, rat growth hormone; RXR, retinoid X receptor; T_3, 3,3′,5-triiodothyronine; TRAP, TR-accessory protein; TRE, T_3-response element.

wished to establish whether the dominant negative potency of mutant receptors may be attributed, at least in part, to a preferential binding to DNA and/or RXRβ, as well as to elucidate molecular mechanisms for the intraindividual differences in the tissue-resistance of patients with GRTH. While TRE-specific and mutation-specific differences in the DNA-binding and transcriptional characteristics were found, we also demonstrated that competition for limiting amounts of RXRβ did not account for the dominant negative mechanism.

Methods

Construction of rRXRβ expression vector. The full-length cDNA for the rat RXRβ was a kind gift of Dr. C. K. Glass, University of California (San Diego, CA) (12). After excising the rRXRβ cDNA from pKS-RXRβ with EcoRI/BstXI, the ends were blunt-ended with T4 DNA polymerase. HindIII linkers were added and the fragment was ligated into the HindIII site of the pSV2 eucaryotic expression vector (9). The construct was verified by restriction analyses.

In vitro transcription and translation of receptors. The construction of pGEM3 vectors containing the mutant cDNAs for the h-TRβ1 from kindreds ED and PV was described elsewhere (9). The vector carrying mutant cDNA from kindred OK was constructed by cloning exon 10 of the h-TRβ1 after amplification by the polymerase chain reaction from genomic DNA into the BglII/HindIII site of pGEM3-h-TRβ1-WT. The subcloned fragment was sequenced to rule out artifactual mutations. The vector containing the normal placental h-TRβ1 cDNA was a kind gift of Dr. C. Weinberger (Salk Institute, San Diego, CA) (24). For in vitro transcription, each cDNA was linearized with HindIII and transcribed with T7 RNA polymerase. [^{35}S]methionine labeled and unlabeled receptors were synthesized using rabbit reticulocyte lysate (Promega, Madison, WI). The labeled translation products were analyzed for the appropriate size, as well as for quantitation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The values were in excellent agreement with the quantitation of [^{35}S]methionine labeled proteins by the trichloroacetic acid precipitation method (3).

Preparation of RXRβ protein. The protein was obtained by infecting SF9-cells with a recombinant baculovirus (rH-2RIIBP) harboring a mouse RXRβ (mRXRβ-H-2RIIBP) cDNA, kindly provided by Dr. Keiko Ozato (National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD). The SF9 cells were cultured in suspension in the serum-free medium SF-900 II SFM (Gibco Life Technologies Inc., Gaithersburg, MD) and infected with rH-2RIIBP virus with a moi of 2 for 2 d at 27°C. The nuclear extracts were prepared according to Dignam et al. (25) except that the protease inhibitors were included in all buffers (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 mM phenylmethyl-sulfonylfluoride). From 1×10^8 SF9 cells, 1.5 ml (5 mg/ml protein) of nuclear extracts were obtained. Analysis by gel electrophoresis indicated that RXRβ accounted for 20–25% of total proteins. The nuclear extracts of wild type SF9 cells were prepared similarly.

Electrophoretic gel mobility shift assay. Single stranded oligonucleotides with sequences shown in Table I were synthesized and purified by high-pressure liquid chromatography. The purified complementary strands were annealed and the ends filled in using Klenow DNA polymerase (Stratagene, San Diego, CA) in the presence of [^{32}P]CTP. The labeled double-stranded oligonucleotides were separated on a 12% polyacrylamide gel. The radioactive band was excised and electrotransferred onto DE 81 paper (Whatman Ltd., Maidstone, United Kingdom). The paper was washed with a low salt buffer (10 mM Tris/pH 7.5, 1 mM EDTA, and 0.1 M LiCl), and thereafter, the labeled oligonucleotides were eluted by a high salt buffer (10 mM Tris/pH 7.5, 1 mM EDTA, and 1 M LiCl). After ethanol precipitation, the labeled oligomers were dissolved in a buffer containing 10 mM Tris/pH 7.5 and 1 mM EDTA and stored at −20°C.

The gel mobility shift assay was carried out similarly as described by Yen et al. (26), except that poly(dI-dC) and bovine serum albumin were omitted, and the concentration of the sheared salmon DNA was increased to 0.8 μg/μl. Briefly, equal amounts of either in vitro–translated wild type or mutant receptor (1–2 μl) were first incubated with the labeled probe (10–12,000 cpm) with or without T_3 in the binding buffer containing 25 mM Hepes/pH 7.5, 5 mM $MgCl_2$, 4 mM EDTA, 10 mM DTT, 0.11 M NaCl, and 0.8 μg/μl sheared salmon DNA). Where appropriate, RXRβ (200 ng of SF9 nuclear extracts) was added. For the control experiments, the nuclear extracts of SF9 cells (200 ng) were added. After incubation for 30 min at 25°C, the mixture was loaded onto a 5.2% polyacrylamide gel and electrophoresed at 4°C for 2–3 h at a constant voltage of 230 V. The gel was dried and subsequently autoradiographed.

Avidin biotin complex DNA binding (ABCD) assay. The protocol is a modification of a previously published method (27). 2–4 μl of reticulocyte lysate containing [^{35}S]methionine-labeled receptors were incubated for 40 min at 4°C with 1–200 ng of biotinylated oligonucleotide in a buffer containing 50 mM KCl, 20 mM Hepes pH 7.8, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Nonidet-40, and 20% glycerol (vol/vol). An aliquot of the incubation mixture was transferred to a tube containing 30 μl streptavidin-agarose slurry. After a 10-min incubation at 4°C, the streptavidin-agarose was quickly washed three times with the above binding buffer at 4°C. The pellet was resuspended in scintillation cocktail and counted.

Transient transfection assay. The construction of the wild-type h-TRβ1 and mutant pSV2 expression vectors, as well as the chloramphenicol acetyltransferase reporter plasmid containing a TREpal (pMTV-TREpal-CAT) was described before (9, 28). The pSV2-OK plasmid was obtained by subcloning the insert from pGEM3-OK into the pSV2-WT vector. The pMTV-TRElap-CAT and pMTV-DR+4-CAT plasmids were constructed by cloning a TRElap (5′-AGCTTGACCTGACGTCAGGTCAAGCTT-3′) or DR+4 (5′-AGCTTAGGTCACTGGAGGTCAAGCTT-3′) oligonucleotide into the HindIII site of pMTV-CAT. The constructs were verified by restriction analyses and sequencing.

Table I. Oligonucleotides Used in the DNA-Binding Experiments

TRE	Sequence
rGH	5′ AAGGGGATCAGGTAAGATCAGGGACGCGACCGCAGG TCCATTCTAGTCCCTGCGCTGGCGTCCTCTAGAAGGA 5′
TREpal	5′ AAGGGGATCCAGCTTCAGGTCATGACCTGAGAGCT TCCAGTACTGGACTCTCGATCTAGAAGGA 5′
DR + 4	5′ AAGGGGATCCAGCTTCAGGTCACAGGAGGTCAGAGAGCT TCGAAGTCCAGTGTCCTCCAGTCTCTCGACTAGAAGGA 5′
TRElap	5′ AAGGGGATCCAGCTTGACCTGACGTCAGGTCAAGCT TCGAACTGGACTGCAGTCCAGTTCGACTAGAAGGA 5′

Table II. Classification of Mutant h-TRβ1

Mutation (kindred/codon)	AA changed	T_3-binding (relative K_d)	Transcription capacity, dominant negative potency	Restoration of h-TR activity by T_3
h-TRβ1-WT	—	1	Normal/none	—
Type I mutation				
ED/312	Ala/Thr	0.2	Reduced/yes	Full
Type II mutation				
OK/437	Met/Val	0.2	Reduced/yes	Partial
Type III mutation				
PV/443	Frame shift	<0.01	Absent/yes	Absent

Cells (CV-1, HeLa) were plated 24 h before transfection in Dulbecco's modified Eagle's medium containing 10% (vol/vol) hormone-depleted fetal calf serum (29), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in 10-cm petri dishes (HeLa) or six-well plates (CV-1) at a density of 1.3×10^6 cells/dish and 0.5×10^6 cells/well, respectively. The medium was changed 4 h before transfection. Using the calcium-phosphate method (CellPhect kit; Pharmacia-LKB, Piscataway, NJ) the cells were transfected with the appropriate plasmids. 24 h later, the plates were washed once with phosphate-buffered saline, and fresh medium was added together with the appropriate T_3 concentration. After another 24 h, the cells were harvested, lysed, and the chloramphenicol acetyltransferase activity determined in the extract as described (30). Chloramphenicol acetyltransferase activity was normalized for the protein concentration as measured by the Coomassie blue method. No substantial differences in the transfection efficiency were present as assessed by the cotransfection of a growth hormone expression vector (pXGH5).

Statistics. Where not indicated otherwise, results were expressed as means±SEM. To examine statistical significance of the Scatchard analysis data in Fig. 8, the slopes of the least square regression lines (with and without T_3) were compared by multiple regression analysis with a categorical variable and using an interaction term (SYSTAT 5.03; Systat Inc., Evanston, IL).

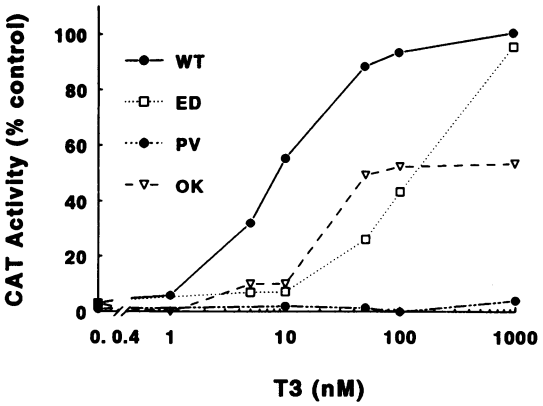

Figure 1. T_3-dependent transcriptional responses of wild type (*WT*) and mutant β1 T_3 receptors assessed by CAT activity. After transfection of HeLa cells (10-cm dishes) with 1 μg of the appropriate receptor expression vector for each kindred and with 5 μg pMTV-TREpal-CAT reporter/plate, the cells were cultured for 24 h in the presence of various T_3 concentrations. The results were normalized to the CAT activity observed with normal h-TRβ1 at 100 nM T_3.

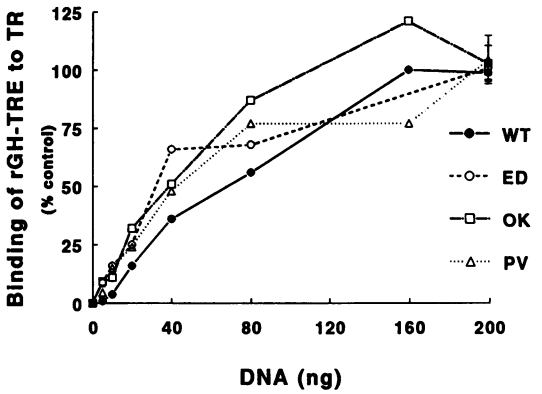

Figure 2. Binding of wild type (*WT*) and mutant h-TRβ1 to rGH-TRE (−186/−158). Equal amounts of in vitro–translated receptors were bound to various amounts of biotinylated rGH-TRE. Similar results were obtained in the absence or presence of 100 nM T_3.

Results

Transcriptional capacity of h-TRβ1-OK. The characteristics of the mutant receptors are summarized in Table II (5). The transcriptional capacity of the mutant receptors on a TREpal in response to T_3 is depicted in Fig. 1. While the T_3-response of the receptors from kindreds ED and PV representing type I and III mutations were reported previously (9), this figure describes the novel functional characteristics of the h-TRβ1 from kindred OK, whose mutation was published recently (5). The transcriptional function of all previously studied mutant h-TRβ1 with residual T_3-binding could be fully restored by high

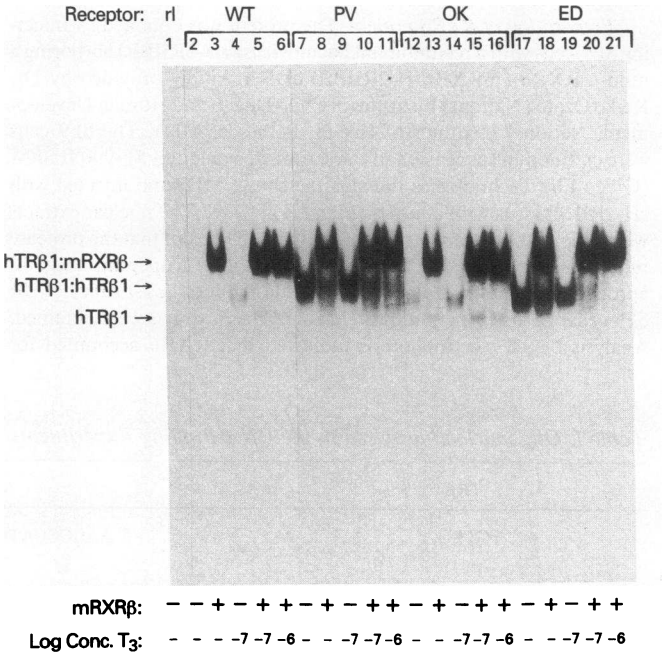

Figure 3. Effect of T_3 and RXRβ on h-TRβ1 binding to TREpal. Equal amounts of in vitro translated receptors were bound to ^{32}P-labeled TREpal oligonucleotide in the presence or absence of T_3 and mRXRβ and then electrophoresed as described in Methods.

levels of T_3, while the mutant h-TRβ1-OK could not achieve full transcriptional activity. In addition, the dominant negative potency of h-TRβ1-OK could not be reversed by high levels of T_3 (data not shown). This suggests that the single point mutation in this kindred impairs independently a T_3-binding domain, as well as a trans-activating domain (type II mutation).

Binding of mutant h-TR to rat growth hormone (rGH) (−186/−158), TREpal, DR+4, and TRElap. The binding of mutant TRs to the rGH promoter (−186/−158) assessed by the ABCD assay is shown in Fig. 2. The Scatchard analysis of the binding curves revealed a small increase in the DNA-binding affinity of all mutant receptors studied (K_d: wildtype, 2.4±0.4 nM; OK, 1.5±0.2 nM; ED, 1.5±0.5 nM; and PV, 1.2±0.2 nM). The binding of TRs to the rGH was not influenced by T_3 at concentrations ≤ 100 nM (data not shown).

In the gel mobility shift assay using the TREpal (Fig. 3) or DR+4 (Fig. 4) as a probe, mutant receptors showed an increased tendency to form homodimers, as summarized in Table III. While T_3 did not alter the binding of normal and mutant TRs to the TREpal, a small decrease (20%) of wild-type homodimer binding was observed on the DR+4 at high levels of T_3 (Fig. 4, *A* and *B*). RXRβ equally heterodimerized with normal and mutant h-TRβ1 and T_3 did not influence this interaction.

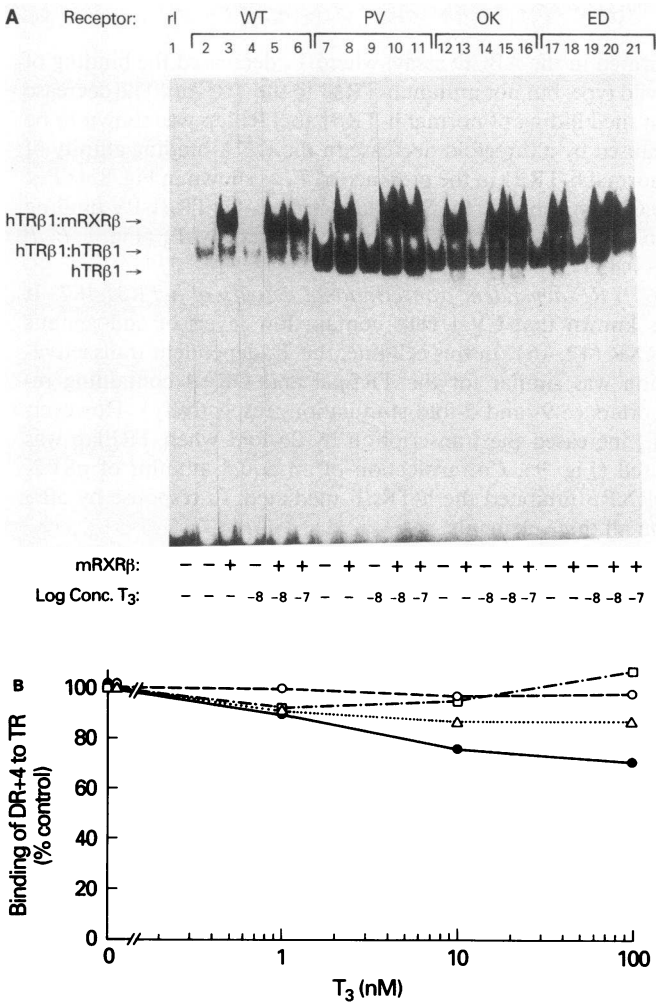

Figure 4. Effect of T_3 and RXRβ on h-TRβ1 binding to DR + 4. Equal amounts of in vitro–translated h-TRβ1 proteins were bound to ^{32}P-DR+4 oligonucleotide in the presence or absence of T_3 and mRXRβ and electrophoresed as described in Methods. (*A*) Autoradiogram. (*B*) After electrophoresis the appropriate dimer bands from four independent experiments were quantitated. Shown is the effect of T_3 on the binding of wild type (●), OK (△), ED (□), and PV (○) h-TRβ1 to DR+4 in the absence of RXRβ. The data are averages of four experiments.

*Table III. Homodimer Formation of WT and Mutant hTRβ1**

	Relative binding			
TRE	WT	PV	ED	OK
PAL	1	5	4	1.5
DR + 4	1	2.3	2	1.5
LAP	1	4	2.8	1.5

* Data were averages of four independent experiments.

Yen et al. have recently shown that on the TRElap derived from the chicken lysozyme silencer (31), T_3 decreased TRα1 and TRβ1 homodimer binding in a dose dependent manner (26). Fig. 5 illustrates the binding of normal and mutant h-TRβ1 to TRElap in the presence of various concentrations of T_3, as well as RXRβ. Addition of nanomolar concentrations of T_3 dissociated the WT homodimers very effectively (Fig. 6 *A*). However, no effect of T_3 on the binding of h-TRβ1-PV, which is characterized by a non-detectable T_3-binding affinity, was observed. The mutants with detectable, but reduced T_3-binding showed a moderate degree of dissociation at high levels of T_3. When RXRβ was added, the normal h-TRβ1 forms a heterodimer (TR:RXR) and a weaker homodimer (TR:TR) band. Upon addition of T_3, the homodimer band further decreases while the heterodimer species increased in a dose-dependent manner (Fig. 6 *B*). This increase in the relative amount of heterodimers was virtually absent with the mutant h-TRβ1. The possibility of h-TRβ1 interacting with an endoge-

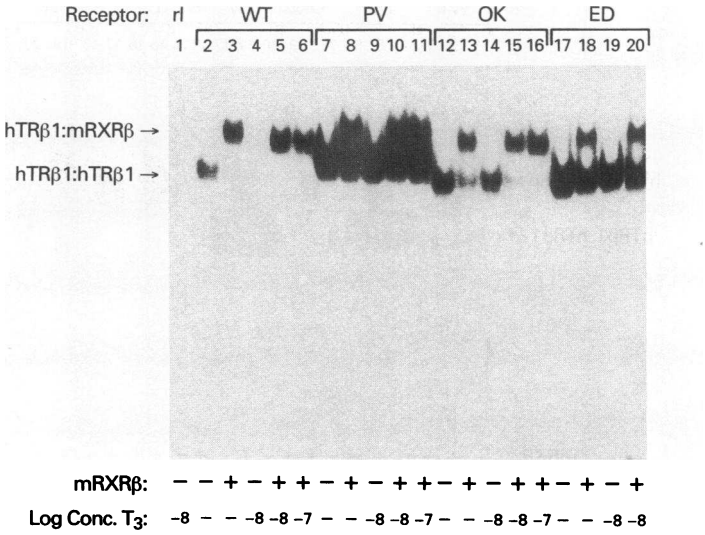

Figure 5. Effect of T_3 and RXRβ on h-TRβ1 binding to TRElap. Equal amounts of in vitro–translated receptors were bound to ^{32}P-labeled TRElap oligonucleotide in the presence or absence of T_3 and mRXRβ and then electrophoresed as described in Methods.

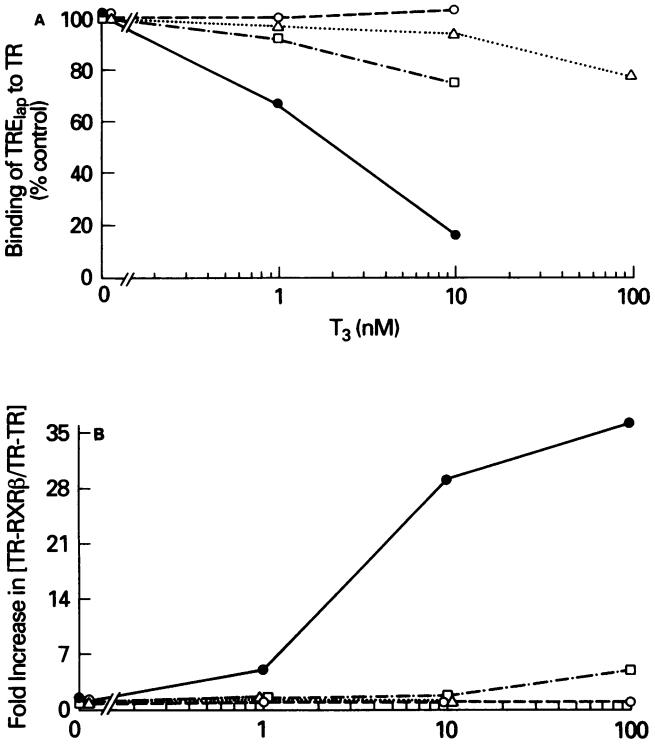

Figure 6. Quantitation of three independent experiments similar to that described in Fig. 5. (A) Effect of T_3 on h-TRβ1 homodimer binding to TRElap in the absence of RXRβ. (B) Effect of T_3 on the ratio of [h-TRβ1:RXRβ] heterodimers to [h-TRβ1:h-TRβ1] homodimers; wild type (●), OK (□), ED (△), and PV (○).

nous SF9 protein was excluded. Fig. 7 shows that in lanes *3, 6, 9,* and *12* for TRElap and lanes *14, 16, 18,* and *20* for DR+4, no heterodimer was detected when the nuclear extracts from the wild-type SF9 cells was used. The above results were con-

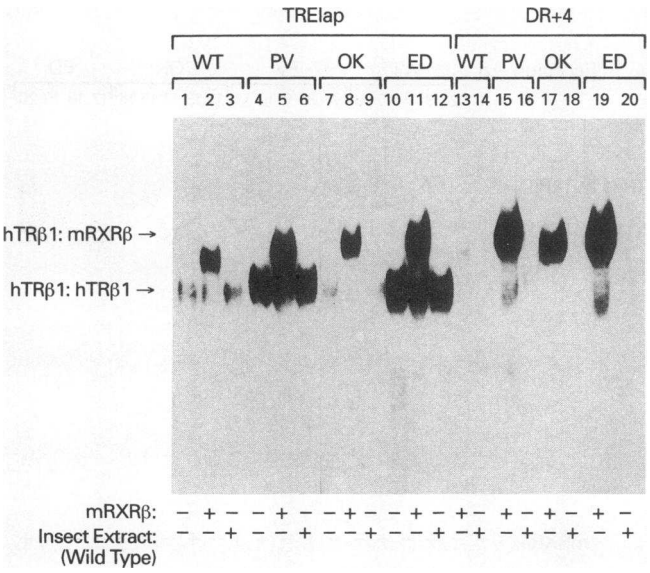

Figure 7. Lack of interaction of SF9 cellular extract with in vitro translated h-TRβ1 protein on TRElap and DR+4. Equal amounts of in vitro–translated receptors were bound to ^{32}P-labeled TRElap and DR+4 in the presence of an equal amount of nuclear extracts of wild type SF9 cells or SF9 cells expressing RXRβ.

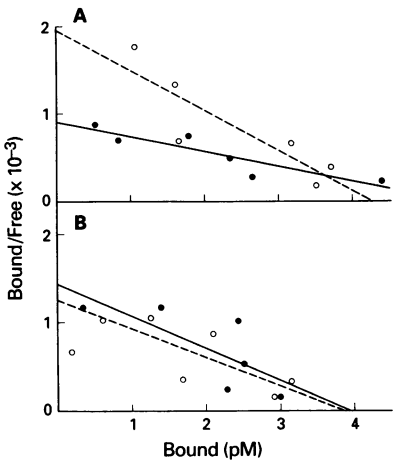

Figure 8. Determination of the dissociation constant of wild type and mutant h-TRβ1 to TRElap using the ABCD assay and scatchard analysis. (A) Binding of wild type receptor in the presence (●) and absence (○) of 100 nM T_3. The affinity of wild type h-TRβ1 is threefold higher in the absence of T_3 (K_d = 2nM) than in its presence (K_d = 6nM; $P \leq 0.05$). (B) Binding of h-TRβ1-PV in the presence (●) and absence (○) of 100 nM T_3. No significant difference exists between the slopes of the two binding curves.

firmed in the ABCD assay, where T_3 decreased the binding of wild type, but not mutant h-TRβ1 to the TRElap. The decrease in the binding of normal h-TRβ1 to TRElap was shown to be caused by a threefold decrease in the DNA-binding affinity of normal h-TRβ1 in the presence of T_3 as shown in Fig. 8 *A* ($P \leq 0.05$). In contrast, the Scatchard curves of h-TRβ1-PV binding to TRElap were not significantly altered by T_3 (Fig. 8 *B*, $P > 0.45$).

TRE-dependent transcriptional activity of h-TRβ1-WT. It is known that CV-1 cells contain low levels of endogenous RXR (12, 16). In this cell line, the T_3-dependent transactivation was similar for the TREpal and DR+4 containing reporters (5.9- and 5-fold stimulation, respectively). However, T_3 increased the transcription by 25-fold when TRElap was used (Fig. 9). Cotransfection of an equal amount of pSV2-rRXRβ inhibited the h-TRβ1–mediated T_3 response by 50% on all three elements.

TRE-dependent dominant negative potency and effect of RXRβ. To explain the dominant negative potency of mutant TRs, it has been proposed that mutant and normal receptors may compete for limiting amounts of a nuclear accessory fac-

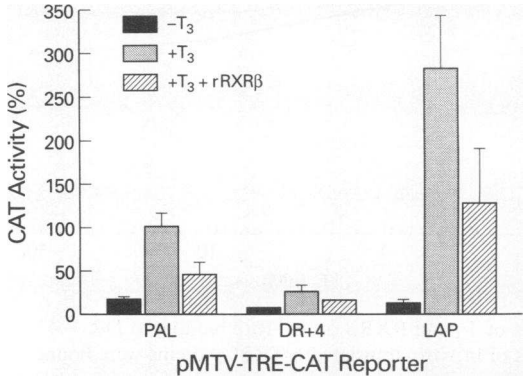

Figure 9. Transcriptional capacity of transfected wild type h-TRβ1 on TREpal, DR+4, and TRElap in the presence or absence of rRXRβ in CV1 cells. pSV2-WT (200 ng) and 1,000 ng of the appropriate pMTV-CAT reporter were transfected with or without 200 ng pSV2-rRXRβ into CV-1 cells in six-well plates. The results were normalized to the CAT activity from h-TRβ1-WT in the presence of 500 nM T_3.

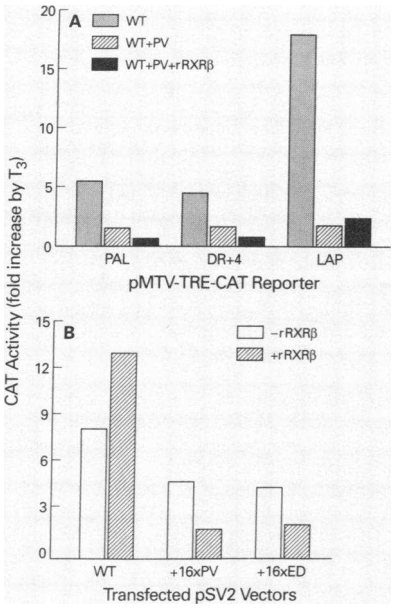

Figure 10. Transfection of rRXRβ does not reverse the TRE-dependent dominant negative potency of mutant h-TRβ1. (A) CV-1 cells in six-well plates were transfected with pSV2-WT (200 ng) alone or with a fivefold excess of pSV2-PV in the presence or absence of 1 μg pSV2-rRXRβ. The results are expressed as fold increase of CAT activity in response to T_3. (B) HeLa cells in 10-cm dishes were transfected with pSV2-WT (250 ng) and pMTV-TREpal-CAT (5 μg) alone or with a 16-fold excess of pSV2-PV or pSV2-ED in the presence (*hatched bars*) or absence (*open bars*) of 5 μg pSV2-rRXRβ. The results are expressed as fold increase of CAT activity in response to T_3.

tor (32). Since the h-TRβ1 mutants were able to interact with RXRβ as discussed above, and RXRβ was shown to account for most of the TRAP activity in HeLa cells, this hypothesis could be directly tested. Transfection of a fivefold excess of h-TRβ1-PV expression plasmid into CV-1 cells resulted in a fourfold decrease in T_3-dependent transcription on the TREpal and DR+4. In contrast, ligand dependent transcription on the TRElap was inhibited by 10-fold (Fig. 10 *A*), thereby establishing the TRE-dependence of the dominant negative potency of mutant h-TRs. Cotransfection of rRXRβ did not reverse this inhibition. Similarly, as shown for HeLa cells in Fig. 10 *B*, the transfection of 5 μg pSV2-rRXRβ increased slightly the transcriptional capacity of normal h-TRβ1 in response to T_3 on the TREpal, whereas rRXRβ was also not able to reverse the dominant negative effect of the mutant TRs from kindreds PV and ED.

Discussion

We investigated the interaction of naturally occurring h-TRβ1 mutants with various TREs and RXRβ. The three different mutations from kindreds with GRTH were chosen to represent three distinct types of functional impairment. The mutant receptor from kindred ED was previously shown to have a fivefold reduction in T_3-binding affinity and a correspondingly shifted T_3-dose response curve in a transient transfection assay; as expected, at high levels of T_3 normal receptor function was restored and the dominant negative potency was abolished (type I mutation) (9). Although this mutation is located in the τ_i subdomain that is thought to be involved in the interaction with TRAPs (33, 34), the h-TRβ1-ED protein interacted normally with RXRβ. The point mutation in the h-TRβ1 of kindred OK reduced T_3-binding also fivefold, but high levels of T_3 could neither restore full receptor activity nor reverse the dominant negative potency (type II mutation) (10). Although a defect in the dimerization with RXRβ might explain these characteristics, we now show that this is not the case. Therefore, it can be speculated that this single mutation not only affects the T_3-binding, but also a trans-activating domain. The type III mutation studied, h-TRβ1-PV, had a frame-shift mutation in the carboxy terminus of the molecule, which is thought to form an amphipathic alpha helix involved in protein–protein contacts (35, 36). Nevertheless, this mutation which did neither bind T_3 nor trans-activate, could interact normally with RXRβ. Together with a report that RXRβ may be a limiting nuclear factor for TR action (15, 37), these findings suggested, that the binding to RXRβ might be crucial for the mediation of the dominant negative action of these receptors. The hypothesis of competition of mutant and normal h-TRs for limiting accessory factors has been proposed before (8, 9, 32). However, we now report that in a transient transfection system using cell lines with low (CV-1) and high (HeLa) levels of endogenous RXR (12, 14, 16), the cotransfection of various amounts of RXRβ could not reverse the dominant negative effect of mutant h-TRβ1.

The DNA-binding affinity of all three mutant receptors was slightly increased on the rGH promoter. Similarly, these mutants demonstrated a variably increased tendency for homodimerization on the three TREs examined in gel-shift experiments. The degree of increased homodimer formation was not dependent on the type of TRE, but was rather influenced by the different mutations in the h-TRβ1; the frame-shifted h-TRβ1-PV exhibited the most pronounced increase in homodimerization. The influence of T_3 on homodimer formation was dependent on the TRE. While ligand did not influence h-TRβ1 binding to TREpal, a small or a marked effect was observed on the DR+4 or TRElap, respectively. The binding of TRs to the latter was previously shown to be T_3-dependent by Yen et al. (26). Data from the same group suggested that a type III mutation in codon 345 (kindred Mf) did not dissociate from TRElap in response to T_3 (38). This observation was confirmed for the h-TRβ1-PV using the ABCD assay, where the binding of ligand to normal h-TRβ1 decreased the binding affinity for TRElap by threefold, while the affinity of h-TRβ1-PV remained unchanged. The type I and II mutations, however, were able to partially dissociate from TRElap in the presence of high levels of T_3. In contrast, T_3 could only minimally increase the RXR:TR heterodimer to TR:TR homodimer ratio for these two types of mutations.

It has recently been shown that DNA-binding is required for the dominant negative effect of certain mutations to occur (11). The increased tendency for homodimerization as found in the present study may render the mutant receptors more efficient competitors on dimer-permissive TREs. It can be hypothesized that the dominant negative potency on TRElap-like enhancers such as the myelin basic protein TRE (26, 31, 39–41), is even more pronounced because of the lack of ligand-induced dissociation of homodimers. This hypothesis was tested and confirmed by transfection studies. While the T_3-dependent transcriptional activity of wild-type h-TRβ1 was fivefold higher on the TRElap than on TREpal or DR+4, cotransfection of mutant h-TRβ1 repressed T_3-dependent trans-activation 2.5-fold more efficiently on the TRElap than on the TREpal or DR+4.

Taken together, our observations suggest that mutant h-TRβ1 inhibit the action of wild-type h-TR mainly by competing for DNA-binding. On TREs where the dissociation of the

TRs is ligand induced, the mutant h-TRβ1 are more effective dominant negative regulators, since they have a higher affinity for the TRE in the presence of T_3 than the wild-type h-TRβ1. While we used the TREpal, DR+4, and TRElap elements as models to study the presumably different binding arrangements of h-TRβ1:RXRβ homo- and heterodimers on DNA (head-to-head, head-to-tail, and tail-to-tail, respectively), it can be speculated that endogenous TREs represent quantitative variations of these themes, subject to the same basic rules established in the present study.

In summary, the functional characteristics of a particular mutant h-TR are not only determined by the type of the mutation itself (type I, II, or III), but in addition by the type of the T_3-regulated enhancer these receptors are acting on. Assuming that the TREs in the promoter of various organ-specific genes are of different types, it is tempting to speculate that the clinically observed intraindividual differences in organ resistance could be explained in part by the TRE-dependent interaction of mutant receptors with DNA and RXRβ.

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