Mutations of CpG Dinucleotides Located in the Triiodothyronine (T_3)-binding Domain of the Thyroid Hormone Receptor (TR) β Gene That Appears to Be Devoid of Natural Mutations May Not Be Detected because They Are Unlikely to Produce the Clinical Phenotype of Resistance to Thyroid Hormone

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Abstract

Thyroid hormone receptor (TR) β gene mutations identified in patients with resistance to thyroid hormone (RTH) revealed two clusters ("hot" areas) of mutations (RTHmut) in the triiodothyronine (T_3)-binding domain. Furthermore, 45% of RTHmuts and 90% of recurring mutations are located in CpG dinucleotides ("hot spots"). To investigate why the region between the two hot areas lacks RTHmuts, we produced 10 artificial mutant TRβs (ARTmut) in this "cold" region according to the hot spot rule (C → T or G → A substitutions in CpGs). The properties of ARTmuts were compared with those of six RTHmuts. Among all RTHmuts, R320H manifesting a mild form of RTH showed the least impairment of T_3-binding affinity (K_a). In contrast, K_a was normal in six ARTmuts (group A), reduced to a lesser extent than R320H in three (group B), and one that was truncated (R410X) did not bind T_3. All RTHmuts had impaired ability to transactivate T_3-responsive elements and exhibited a strong dominant negative effect on cotransfected wild-type TRβ. Group B and A ARTmuts had minimally impaired or normal transactivation and weak or no dominant negative effect, respectively. R410X showed neither transactivation nor dominant negative effect. Natural mutations expected to occur in the cold region of TRβ should fail to manifest as RTH (group A) or should escape detection (group B) since the serum thyroid hormone levels required to compensate for the reduced binding affinity should be inferior to those found in subjects with R320H. R410X would manifest RTH only in the homozygote state. The cold region of the putative T_3-binding domain is relatively insensitive to amino acid changes and, thus, may not be involved in a direct interaction with T_3. (*J. Clin. Invest.* 1994. 94:607–615.) Key words: dominant negative effect • transactivation • resistance to thyroid hormone • CpG dinucleotides • thyroid hormone response element

Introduction

Resistance to thyroid hormone (RTH)[1] is a syndrome characterized by a variable tissue hyposensitivity to thyroid hormone. Since the diagnosis of RTH is based principally on the presence of high free thyroid hormone levels in serum associated with normal or slightly elevated thyrotropin (TSH) concentration, the pituitary thyrotrophs are invariably involved in the hormone resistance. Goiter and manifestations suggestive of thyroid hormone deficiency or excess reflect the imperfect compensatory mechanism through increased synthesis and secretion of thyroid hormone. These have been reviewed in detail recently (1).

In 1986, two thyroid hormone receptor (TR) cDNAs, the TRα and TRβ, were cloned (2, 3) and were shown to belong to the nuclear receptor superfamily, which includes the receptors for steroid hormones, vitamin D, retinoic acid, and other unidentified ligands (4). This breakthrough led to the demonstration of linkage between RTH and the TRβ gene (5), which was followed rapidly by identification of mutations in exons 9 (6) and 10 (7) of two unrelated families. 30 different mutations have been identified so far in 40 families (8–25) (Fig. 1). Most are point mutations located in the COOH-terminal half of the molecule which contains part of the hinge domain (D domain) and the ligand-binding domain (E domain). The resulting TRβs have decreased affinity for 3,5,3′-triiodothyronine (T_3) and impaired transactivation in transient transfection assays based on reporter genes linked to thyroid hormone response elements (TREs). More importantly, these mutant receptors dominantly inhibit the transactivation function of cotransfected wild-type (WT) TRs, accounting for the dominant pattern of RTH inheritance (dominant negative effect) (12, 26–28).

The growing list of mutant TRβ genes found in subjects with RTH lead to the identification of two mutational "hot" areas in the T_3-binding domain of the TRβ. One of these two areas spans from codon 310 to 347 and the other from codon 438 to 459, the latter located two amino acids upstream of the carboxy terminus (11, 13, 14, 21). They occur with high frequency in CG-rich sequences (21). Not a single mutation has been identified so far in the region between the two hot areas. This "cold" region, encoding 90 amino acids, contains eight

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1. *Abbreviations used in this paper:* ARTmut, artificial mutant; CAT, chloramphenicol acetyl transferase; F2, chicken lysozyme silencer; h, human; K_a, association constant; lap, inverted palindrome; Luc, firefly luciferase; pal, palindrome; RTH, resistance to thyroid hormone; RTHmut, natural mutations associated with RTH; RXR, retinoid X receptor; TR, thyroid hormone receptor; TRE, thyroid hormone responsive element; WT, wild-type.

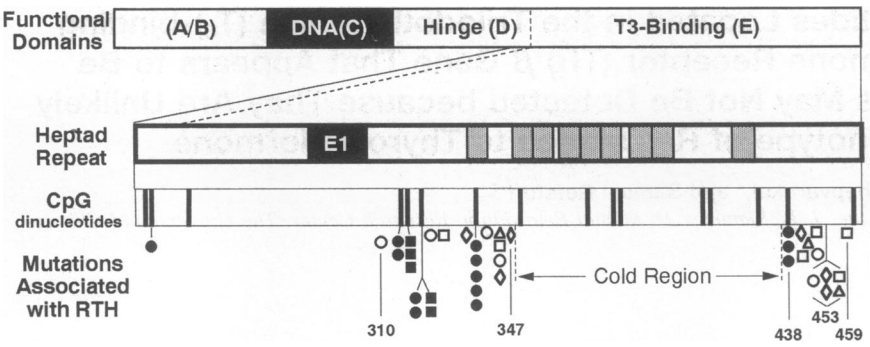

Figure 1. Functional structure of TRβ and location of CpGs and natural mutations identified in subjects with RTH. On top of the figure is a diagrammatic representation of the putative functional domains of TRβ. The COOH-terminal half, which includes part of hinge domain (D) and T_3-binding domain (E), is enlarged below and shows the E1 subdomain (30, 31) and hydrophobic heptad repeats (29), indicated by dotted boxes. Location of mutations (*symbols*) in families with RTH are shown in the lower portion of the figure along with the location of CpG dinucleotides (*bars*). Identical symbols lined up vertically represent the same mutation reported in unrelated families. Closed symbols represent C → T or G → A substitutions in CpGs. Other mutations are indicated as open symbols, and those lined vertically but denoted by different symbols represent different nucleotide substitutions in the same codon. The cold region is indicated, and numbers are those of amino acids.

of the nine heptad repeats, described by Forman et al. (29), which appear to play an important role in receptor dimerization (Fig. 1).

TREs are composed of two or more AGGTCA-like half site motifs, which are also recognized by other members of the nuclear receptor superfamily including retinoic acid and vitamin D receptors. They are arranged in palindromes (pal), inverted palindromes (lap), or direct repeats with 4-bp spacing. TRs can bind to these TREs as monomers, homodimers, or heterodimers with TR auxiliary proteins (32, 33), such as the retinoid X receptor (RXR) (34). Monomeric binding is relatively weak and nonspecific compared with dimeric binding (35). Thus, it can be postulated that, by preventing TR dimerization, mutations in the cold region would produce TRβs with reduced dominant negative effect because they can neither titrate out TR auxiliary proteins nor compete for DNA-binding sites effectively. Such mutations would escape detection as do heterozygotes with TRβ gene deletion (12). Indeed, an earlier study has shown that an artificial TR mutant, which was unable to dimerize, lost its inhibitory effect on the transactivation of retinoic acid receptor (36). Furthermore, recent studies showed that heterodimerization of mutant TRs with RXR is important in mediating their dominant negative effect (37, 38). However, in these reports, mutations were specifically designed to destroy hydrophobic residues, resulting in patterns of amino acid substitution quite different from those found in naturally occurring mutations. Moreover, while the function of the ninth heptad repeat (residue 421–428) (29) has been studied extensively in these reports (36–38), no systematic studies have been undertaken to determine the function of the cold region, including the second to eighth heptad repeats. Accordingly, the reason for the failure to detect mutations in the cold region of the TRβ gene remains a matter of conjecture.

In this report, we made 10 artificial mutations in CpG dinucleotides located in the cold region of the TRβ gene. Such artificial mutants (ARTmuts) result in patterns of amino acid substitutions similar to those found in naturally occurring mutant TRβs causing RTH (RTHmuts). These ARTmuts were evaluated in terms of T_3-binding, transactivation function, and dominant negative effect and were compared with six RTHmuts. The results suggest that, through preservation of normal function or absence of dominant negative effect, these 10 ARTmuts would produce little, if any, clinical manifestation of RTH and, therefore, would escape detection.

Methods

Cell culture

SV40-transformed African green monkey kidney cells (Cos-1) and human hepatoblastoma cells (HepG2) were grown in DME (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% T_3-stripped (39) FBS at 37°C, 5% CO_2 and 100% humidity.

Construction of the plasmids and site-directed mutagenesis

TRβ expression vectors. The human (h) TRβ cDNA clones pheA4 and pheA12 were spliced, combined, and inserted into the BamHI/EcoRI sites of the mutagenesis vector, pSelect (Promega Corp., Madison, WI), and the mammalian expression vector, pcDNAI/Amp (Invitrogen, San Diego, CA). Mutageneses were performed in pSelect-hTRβ using 31-bp mutagenic oligonucleotides according to the instructions provided by the supplier. After screening for the desired mutation by PCR-mediated restriction fragment length polymorphism,[2] appropriate endonuclease restricted fragments containing the mutations were transferred into corresponding sites of the pcDNAI/Amp-hTRβ. The final constructs were verified by sequencing.

Reporter vectors. TREpal x3-Luc and F2 x3-Luc were constructed by placing the respective TRE tk promoter elements, contained in the TREpal x3 tk CAT and F2aas tk CAT reporter vectors (40) into the multiple cloning site of pGL2-Basic Vector (Promega Corp.) upstream of the firefly luciferase (Luc) fusion gene. TREpal x3 consists of three copies of two consensus TRE hexamer half motifs in palindromic arrangement spaced by 10 nucleotides between each TREpal repeat.[3] F2aas consists of three copies of the chicken lysozyme silencer (F2), a sequence made of two TRE hexamer half sites arranged as an imperfect inverted palindrome (lap) and spaced by six nucleotides in palindromic arrangement between the half motifs. The three F2 sequences are oriented as antisense, antisense, and sense (aas) and their TRElap repeats are spaced by 11 (antisense–antisense) or 8 (antisense–sense) nucleotides.[4]

2. A complete list of mutagenic or screening oligonucleotides will be provided upon request.

3. aagctttcAGGTCATGACCTga. The TREpal sequence is in capital letters, and the spacer sequence is in lowercase letters.

4. ttatTGACCCcagctgAGGTCAagttacg. The TRElap sequence is in capital letters, and the spacer sequences are in lowercase letters.

Receptor expression

Cos-1 cells were transfected with 10 μg of pcDNAI/Amp-TRβ and 10 μg of carrier DNA (Bluescript; Stratagene, La Jolla, CA) by the calcium phosphate coprecipitation method (41). After overnight incubation, cells were washed twice with HBSS (Life Technologies Inc.) and further incubated for 72 h. Then, cells were harvested, and whole cell extracts were prepared according to Damm et al. (42). Alternatively, TRβs were synthesized by in vitro transcription and translation using T_7-coupled TNT lysate (Promega Corp.).

Binding assay

The filter binding assay described by Inoue et al. (43) was used with minor modifications. Briefly, 1–5 μl of whole cell extracts was incubated at 4°C for 18 h with 14 fmol of [^{125}I]T_3 (Du Pont, Boston, MA; 2,200 Ci/mmol) in the presence of 0–7,000 fmol of unlabeled T_3 (Sigma Immunochemicals, St. Louis, MO). The protein-bound fractions were collected onto nitrocellulose membranes and counted. Scatchard analysis was used to determine the T_3 association constants (K_a). Since, in independent binding assays, the K_a of the WT TRβ varied from 1.2×10^{10} to 4.8×10^{10}, K_as of the mutant TRβs were normalized by dividing their value by that of the WT-TRβ K_a which was determined with each assay. Values are expressed as mean±range of K_a mut/K_a WT obtained from at least two independent binding assays.

Transient transfection and luciferase assay

HepG2 cells were propagated in 12-well plastic plates and transfected 24 h later with 1 μg of the reporter vectors, 1 μg of SV2 β-gal, and 0.2 μg of TRβ expression vectors per well. Cells were incubated for 16–20 h with DNA-calcium precipitate, washed twice, and then incubated for an additional 48 h with the complete medium containing T_3-stripped FBS, in the absence or presence of different amounts of T_3. Cells were lysed and assayed for luciferase activity using luciferase assay reagents (Promega Corp.) and Luminometer 2010 (Analytical Luminescence Laboratories, San Diego, CA). β-gal activity was measured with Galacto-Light (Tropix Inc., Bedford, MA) to monitor transfection efficiency. Since variations in transfection efficiency were < 15% in triplicate wells, values are presented as fold induction of luciferase activity by the addition of T_3 without normalization for β-gal activity.

Results

Location of mutations and their effect on T_3-binding. The locations of mutations in the TRβ gene associated with RTH are depicted in Fig. 1. As previous studies have pointed out (11, 13, 14, 21), they cluster in two regions of the putative T_3-binding domain. Mutations most often involve substitutions of cytosines with thymidines (C → T) or guanines with adenines (G → A) in CpG dinucleotides. Cytosines in CpGs are frequently methylated in mammalian cells (44), and deamination of methylated cytosines results in C → T or G → A substitutions that escape the DNA repair mechanism. Since 32% of nucleotide substitutions in human genetic diseases are C → T or G → A in CpGs, they have been designated mutational hot spots (for review see reference 45). 45% of mutations reported in families with RTH and 90% of those identified in multiple unrelated kindreds with RTH follow this mutation rule (Fig. 1).

We produced ARTmuts in the cold region of the TRβ gene, according to the hot spot rule. There are seven CpGs in this cold region spanning codons 348–437 that result in 14 different nucleotide substitutions (Table I). Four produce silent mutations, one produces a nonsense mutation, and nine produce missense mutations. The latter 10 mutations that produce amino acid changes were introduced into the TRβ cDNA by site-directed mutagenesis. Six naturally occurring mutations found in subjects with RTH (RTHmuts) were also constructed. These include the extensively studied mutant Mf (G345R) (6, 26, 27, 46) and five other RTHmuts occurring at CpGs. All the mutant receptors, including R410X, had the predicted size on SDS-PAGE when synthesized in reticulocyte lysate in the presence of [^{35}S]methionine (data not shown). Mutant receptors were also expressed in Cos-1 cells, and their K_as were determined. Table II gives the list of mutant TRβs studied in this report and their relative affinity for T_3 and identifies the families having the six natural mutations.

Table I. Location of CpG Dinucleotides in the Cold Region of the TRβ Gene (Codons 348–437) and the Amino Acid Changes That Result from All Possible Mutations by Methylation–Deamination

351 **352**		**410**	
Asp **Ala**	→ Asp Ala	**Arg**	→ **Stop**
GAC GCC	GAT GCC	CGA	TGA
	→ Asp **Thr**		→ **Gln**
	GAC ACC		CAA
375 **376**		413 **414**	
Ala **Val**	→ Ala Val	His **Val**	→ His Val
GCC GTC	GCT GTC	CAC GTG	CAT GTG
	→ Ala **Ile**		→ His **Met**
	GCC ATC		CAC ATG
383		**429**	
Arg	→ **Cys**	**Arg**	→ **Trp**
CGC	TGC	CGG	TGG
	→ **His**		→ **Gln**
	CAC		CAG
384			
Pro	→ **Leu**		
CCG	CTG		
	→ Pro		
	CCA		

Amino acid replacements are indicated in bold letters, and the CpG dinucleotides and their substitutions are underlined.

Note that five of the six patterns of amino acid substitution found in RTHmuts are represented in the ARTmuts (R → H, W and A → T).

All RTHmuts produced TRβs with > 50% reduction in T_3-binding affinity. The highest T_3 binding among the RTHmuts was 40% that of the WT TRβ. This mutation, R320H, is associated with a relatively mild form of RTH in terms of the free thyroid hormone level required to maintain a normal serum TSH concentration (15, 47). Among the ARTmuts, the nonsense mutation, R410X, lacking 51 COOH-terminal amino acids of the ligand-binding domain, was devoid of T_3-binding activity. To our surprise, however, all other missense mutations in the cold region of the TRβ showed no changes or resulted in only minor alterations in ligand-binding properties (Table II). The T_3-binding affinity of R383C was the lowest among the ARTmuts having a single amino acid substitution but it still had a higher K_a compared with the RTHmut R320H. In general, missense ARTmuts which are closer to the amino terminus (amino acids 352–383) had lower T_3-binding affinity compared with ARTmuts located closer to the carboxy terminus (amino acids 384–429). The T_3-binding affinities of the latter were not significantly different than that of the WT TRβ. V376I is an exception to this rule probably because both valine and isoleucine are hydrophobic amino acids and an additional methyl residue in isoleucine is the only difference with valine. Accordingly, we divide the ARTmuts into two groups and R410X. Group A

Table II. Mutant TRβs Studied in This Communication and Their Affinity for T_3

Mutant TRβ (amino acid substitution)*	K_a mut/K_a WT (mean±range)	Family identification‡	References§
R316H	0.05±0.01‖	GH, F120	22, 22a
A317T	0.16±0.05‖	F52, F89, F100(ED)	21, 13, 11
R320H	0.42±0.02‖	F64(CL), F95	15, 47
R338W	0.21±0.04‖	F29, F106, F112(KT), FE, LT	14, 21, 23, 24
G345R	<0.03‖	F44(Mf)	6
A352T	**0.81±0.09‖**		
V376I	**0.94±0.19**		
R383C	**0.62±0.06‖**		
R383H	**0.72±0.12‖**		
P384L	**1.05±0.16**	**Artifical mutations**	
R410Q	**0.99±0.07**		
R410X	**<0.03‖**		
V414M	**0.95±0.11**		
R429Q	**1.00±0.00**		
R429W	**0.91±0.23**		
R438H	0.25±0.07‖	F45, F68, F111	10, 13, 25

* Amino acids are shown in one letter codes. ‡ Family identification is according to that used in a recent review (1) and/or provided in the original report. § Given in the order of family quoted. ‖ Significantly reduced relative to WT.

consists of those ARTmuts that bind T_3 as well as WT TRβ (V376I, P384L, R410Q, V414M, R429Q, and R429W). Group B comprises A352T, R383C, and R383H with reduced T_3 binding to a level 60–80% of that of the WT TRβ.

Transactivation function of mutant TRs. To test the transactivation function of the 16 mutant TRβs, we constructed two T_3-regulated reporter genes, TREpal x3-Luc and F2 x3-Luc. We chose these reporter vectors, with three copies (x3) of the TREpal or F2, in order to obtain a signal sensitive at low and physiologic T_3 concentrations. The hepatoblastoma-derived cell line, HepG2, was chosen as a host for transient transfection since liver is both a sensitive and an important target organ for T_3 action and because this cell line gave a more consistent level of transfection compared with CV1 and Cos-1 cells.

As shown in Fig. 2, cotransfection of either reporter with the WT-TRβ expression vector resulted in high levels of T_3-sensitive induction of luciferase activity. In the absence of TRβ expression vector, addition of T_3 resulted in only a 20–50% increase in luciferase activity, which was presumably mediated by the endogenous TR in HepG2 cells (data not shown). Note that the T_3–dose-dependent response patterns were different for each of the two TRE-containing reporters. With 10 nM of T_3, TREpal x3-Luc reached maximal induction, while F2 x3-Luc was only partially transactivated. Addition of 100 nM of T_3 induced further activation of F2 x3-Luc, reaching the maximal level of luciferase induction observed with TREpal x3-Luc. This result is in agreement with a recent report by Zavacki et al. (48) showing similar TRE-dependent T_3-dose–response patterns using a single copy of TREpal and F2. Thus, while benefiting from the increased sensitivity to T_3, the TRE-specific transactivation pattern is conserved in multiple tandem TREs. We used these

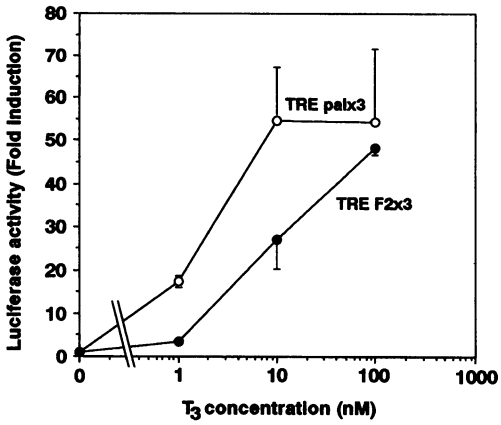

Figure 2. Transactivation patterns of TREpal x3-Luc and F2 x3-Luc. Each of the two reporter vectors was cotransfected with a WT-TRβ expression vector into HepG2 cells. Open symbols represent the magnitude of responses of TREpal x3-Luc, and closed symbols represent those of F2 x3-Luc both at four concentrations of T_3. Values are mean±SD.

two reporter vectors to test T_3-dependent transactivation function of the 16 mutant TRβs.

Transactivation by the WT TRβ and mutant TRβs was tested without and with the addition of 5 nM T_3, and the difference was expressed as fold induction by T_3. As shown in Fig. 3, *A* and *B*, WT TRβ activated on the average TREpal x3-Luc by 43-fold, which is below the 55-fold maximum achieved with the addition of 10 and 100 nM T_3, and F2 x3-Luc by 12-fold which is one-quarter of maximum. 5 nM of T_3 is close to the physiological conditions of 50% T_3-receptor saturation, which is characteristic of endogenous TRs in most tissues (49). The transactivation function of RTHmuts was impaired to various degrees. Three (R316H, A317T, G345R), with T_3 binding < 20% that of WT TRβ, did not transactivate either reporters at 5 nM T_3. On the other hand, R320H, R338W, and R438H, with less severe T_3-binding impairment, showed some transactivation of at least one of the two reporters. Among the RTHmuts, R320H, with T_3-binding 42% that of WT TRβ, showed the strongest transactivation, which is eightfold and twofold on TREpal x3 and F2 x3, respectively.

As expected from the T_3-binding affinity values, all nine missense ARTmuts transactivated both reporters to a greater extent than R320H. Only small differences in the magnitude of transactivation were observed between WT-TRβ and group A mutants (V376I, P384L, R410Q, V414M, R429Q, and R429W). Group B mutants, with 62–81% T_3-binding affinity compared with WT TRβ, produced lower induction of luciferase activity on both reporters, especially F2 x3-Luc. However, the degree of impairment was clearly smaller than that observed with the RTHmuts, including R320H. R410X did not mediate T_3 action at all. As shown in Fig. 3, *C* and *D*, there is a good correlation between T_3 binding and transactivation activity.

Dominant negative inhibition by mutant TRβs. To test the dominant negative effect of mutant TRβ genes, we transfected HepG2 cells with TREpal x3-Luc or F2 x3-Luc together with the expression vectors for WT TRβ and each mutant TRβ. In preliminary experiments with G345R, strong dominant negative effect could be observed when equal amounts of WT TRβ and G345R expression vectors were cotransfected. In most previous

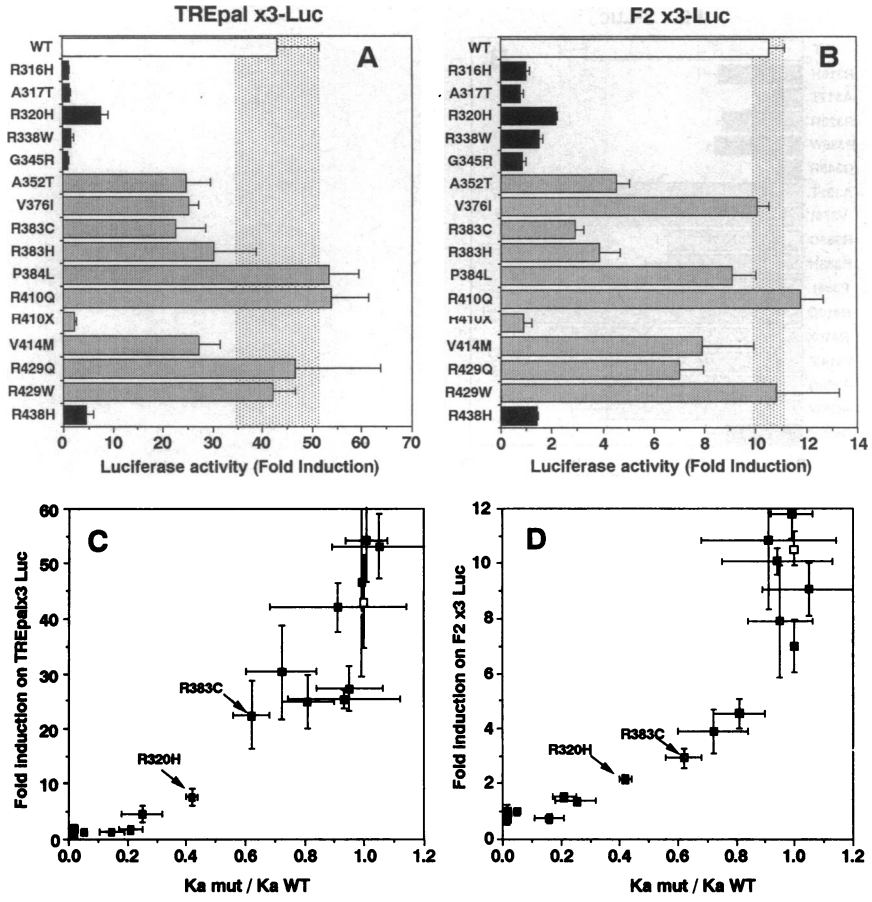

Figure 3. Transactivation function of mutant TRs and its correlation with T_3-binding affinity. HepG2 cells were transfected with TREpal x3-Luc (*A* and *C*) or F2 x3-Luc (*B* and *D*) together with the various TRβ constructs in the expression vector, pcDNAI/Amp. Results are expressed as fold induction of luciferase activity in the presence of 5 nM T_3 relative to that observed without added T_3. Values are mean±range of triplicate experiments. In *C* and *D*, transactivation is plotted against the relative T_3-binding affinity. The latter is expressed as K_a mut/K_a WT, and values are mean±range from at least two independent determinations. WT TRβ is shown as open bars and squares. RTHmuts are in black, and ARTmuts are shaded. 1 SD of WT TRβ is shown as a lightly shaded area.

studies, to demonstrate a dominant negative effect, it was required to transfect mutant TRs in 5- to 10-fold excess amounts relative to the cotransfected WT TR (26–28). Our ability to detect a dominant negative effect with a greater sensitivity is presumably due to the cooperativity of the three copies of TRE sequences used in our reporter vectors. In other terms, one TRE occupied by a mutant TRβ can effectively inhibit the WT TRβ action on the two other coupled TREs. We believe that the one to one ratio of WT TRβ to mutant TRβ expression vectors mimics the physiological condition in the heterozygous individuals with RTH.

Fig. 4, *A* and *B* shows the transactivation activity in the presence of WT TRβ and mutant TRβ vectors. When TREpal x3-Luc was used as reporter, all the RTHmuts inhibited transactivation of the WT TRβ gene by more than 60%. Similar results were observed with the F2 x3-Luc reporter. A317T and G345R, which produced no significant transactivation with 5 nM T_3, showed also the strongest dominant negative effect. On the other hand, three RTHmuts (R320H, R338W and R438H), with T_3-binding affinities of 20–40% of the WT TRβ, were relatively weak inhibitors of transactivation. This weaker inhibition may be accounted for by the small to moderate decrease of T_3-mediated transactivation by these receptors. Indeed, there is a rough correlation between the magnitude of dominant negative effect and the degree of impairment in T_3 binding (Fig. 4, *C* and *D*). Similar results have been reported previously (27, 28). However, R316H, with T_3 binding near the lower limit of detectability and no demonstrable induction of transactivation with 5 nM T_3, exhibited a relatively weak dominant negative effect compared with A317T and G345R.

Compared with RTHmut, ARTmuts showed much weaker or no dominant negative effect. None of the group A ARTmuts, with affinities for T_3 not significantly different than that of WT TRβ, showed consistent dominant negative effect with both reporter genes. This was expected, since they had transactivation activity equal to that of WT TRβ when transfected alone. On the other hand, group B ARTmuts consistently showed a dominant negative effect, albeit of a lesser magnitude than any of the RTHmuts. R410X, which neither bound T_3 nor had transactivation activity, did not show any dominant negative effect whatsoever. This result is in agreement with reports showing that residues 421–428, which form the ninth hydrophobic heptad, are required for interaction with auxiliary proteins and dominant negative inhibition (37, 38).

Discussion

The principal goal of our investigation was to determine why the majority of natural mutations associated with RTH cluster in two hot areas of the T_3-binding domain of the TRβ gene, sparing an intervening cold region of 90 contiguous amino acids (residues 348–437). Two hypotheses were considered: (*a*) mutations in this cold region of the TRβ gene may be rare or not occur at all; or (*b*) mutations occurring in the cold region may not have been detected because they do not produce RTH. The latter hypothesis appeared more plausible to us since this region

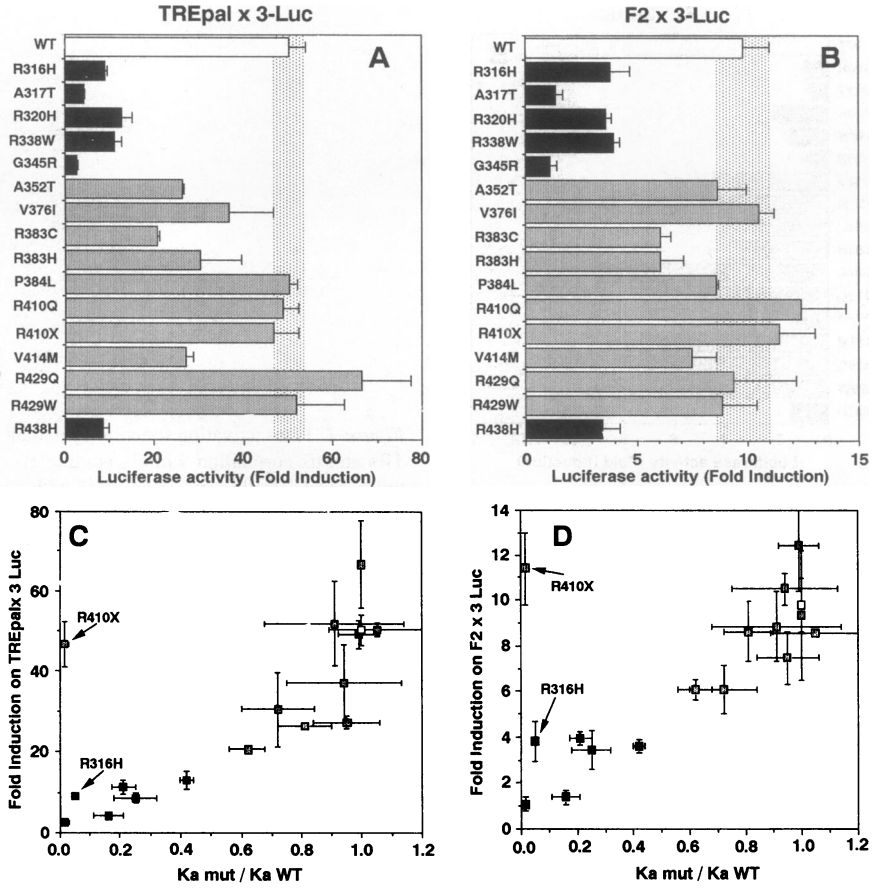

Figure 4. Dominant negative effect of mutant TRs. HepG2 cells were transfected with TREpal x3-Luc (*A* and *C*) or F2 x3-Luc (*B* and *D*) together with pcDNAI/Amp-hTRβ-WT and each of pcDNAI/Amp-mutant hTRβ. The ratio of transfected mutant and WT TRβ was 1:1. Values are mean±range of triplicate experiments. In *C* and *D*, transactivation in the presence of both WT TRβ and mutant TRβ is plotted against the relative T_3-binding affinity of the respective mutant TRβ. WT TRβ is shown as open bars and squares. RTHmuts are in black, and ARTmuts are shaded. 1 SD of WT TRβ is shown as a lightly shaded area.

is not devoid of mutational CpG dinucleotide hot spots and because the same region encodes the putative dimerization domain involved in protein–protein interactions. Preservation of TRβ dimerization is believed to be necessary for the manifestation of RTH in heterozygous subjects, and, thus, it appeared likely that expression of mutant TRβ genes with impaired T_3 binding, as well as impaired dimerization, would behave phenotypically as a silent, null mutation (12).

To test the latter hypothesis, we created artificial point mutations in the cold region of the TRβ gene. In choosing the locations and types of nucleotide substitutions, the following facts were taken into consideration. Of the 40 families with RTH in which mutations in the TRβ gene have been described, 30 (75%) occur in CG-rich segments, and 60% of these involve nucleotide substitutions in CpG dinucleotide hot spots (reference 21 and Fig. 1). This observation is not unique to mutations of the TRβ gene. Indeed, the majority of single base substitutions causing human genetic diseases or DNA polimorphisms follow the hot spot mutation rule of CG to TG and CG to CA transition (45, 50). Taking into consideration all possible nucleotide transitions in the seven CpG dinucleotides present in this cold region (Fig. 1), 10 mutations producing amino acid changes were identified (Table I) and introduced by site-directed mutagenesis. This strategy resulted in the alteration of the following amino acids: A352, V376, R383, P384, R410, V414, and R429. With the exception of P384 and V414, these amino acids are conserved in the TRα and TRβ genes across species including man, rat, mouse, chicken, and frog. R383, R410, and R429 are conserved even in the retinoic acid receptor genes. To our surprise, none of the nine missense mutations introduced in these six residues altered the properties of TRβ to nearly the same extent as those resulting from TRβ mutations found in subjects with RTH.

Except R410X, all other ARTmuts retained a relatively high binding affinity for T_3. A recent report proposed that the structure of the hormone-binding domain consists of an α/β barrel (51). Based on the predicted secondary structure, R383, P384, R410, and V414 are close to maximal in prediction for molecular flexibility. Although such a model might suggest that alterations produced by amino acid changes in the flexible region would have a minor effect on T_3 binding, this does not seem to be the case since several RTHmuts, with severe impairment of T_3 binding, are also located in the flexible region. Furthermore, the ARTmuts R383H and R383C resulted in some impairment of T_3 binding as well as transactivation function and were able to inhibit transactivation mediated by the WT TRβ. On the other hand, and in agreement with the α/β model, mutation of P384, R410, and V414M produced no significant changes in receptor function. The replacement of A352, located in the β strand region, by threonine, produced a 20% reduction in T_3 binding and a proportional impairment in transactivation. In an earlier study by Spanjaard et al. (52), substitution of the homologous alanine residue in TRα, by a proline, resulted in a 50% reduction in T_3 binding and transactivation and, based on the results of ABCD-assay, a slight impairment of heterodimerization as well. Mutation of V376, also located in a β strand

region, produced no alteration in T_3 binding and only minimal decrease in transactivation function only when tested with the reporter TREpal x3-Luc. This may be the result of the similarity between valine and its isoleucine replacement. Finally, most surprising is the failure to observe significant functional changes with the substitution of R429 by glutamine or by tryptophan. This amino acid, located in the helical region of TRβ, is moreover next to L428 whose substitution by arginine results in the loss of T_3 binding and transactivation function despite the concomitant loss of heterodimizeration and dominant negative effect (37, 38).

The mechanisms involved in the dominant inhibition of TRs are not fully understood although the topic has been the subject of extensive investigations. Forman et al. (53) showed that a transcriptionally inactive TR, lacking the DNA-binding domain, has dominant negative effect. This observation suggests that formation of dimers between inactive TRs and WT TRs or squelching of cofactors are likely mechanisms of dominant inhibition. In contrast, another group of investigators (54) showed that intact DNA binding is required for the mediation of the dominant negative effect, proposing that competition for TRE plays a more important role. Using a gel mobility assay, Yen et al. (46) showed that T_3 induced a dissociation of WT TRβ homodimers bound to F2-TRE while this failed to occur with the mutant TRβ, G345R, which binds normally to TRE but does not bind T_3. This finding demonstrates yet another feature of the dominant negative interaction, namely failure to abrogate the inhibitory effect of unliganded TR homodimers and competition for TRE. Moreover, two independent reports (37, 38) showed mutant TRs that bind to DNA as homodimers but not as heterodimers with RXR and cannot mediate an inhibitory effect, posing new questions concerning the physiological relevance of TR homodimers. These reports as well as more recent reports (48, 55) support the notion that the dominant negative effect is not mediated by a single mechanism. Rather, the mode of interaction is specific for the organization and sequences of different TREs. Though, in this report, similar results were obtained with two distinct TREs, interactions of yet unknown nature with other TREs may reveal different effects of the ARTmuts we tested on WT TRs.

It is appropriate to examine the contribution of the current report to the understanding of the RTH syndrome. What can be learned about the relation of TRβ mutations with the clinical manifestations of RTH? Group A ARTmuts, with T_3-binding affinity and in vitro biological function indistinguishable from that of WT TRβ, are expected not to produce any clinical signs of RTH. Even the three ARTmuts that exhibited impairment of function in terms of T_3 binding and transactivation and are, thus, classified as group B are unlikely to cause clinically detectable changes. This conclusion is based on the comparison of R383C, the ARTmut displaying the most pronounced impairment of T_3 binding and function, with two RTHmuts (R320H and R316H) that exhibit the most mild impairment of T_3 binding and clinical manifestations, respectively. R320H, with 40% T_3-binding activity, has been identified in members of two unrelated families (15, 47). In both families, affected members had a minimal elevation of free thyroxine (T_4) and T_3 levels in serum. As a matter of fact, in one of the two families, F95, some affected individuals had serum hormone levels that overlapped with those found in members of the family expressing only the normal TRβ gene (47). Similarly, one affected member of the other kindred expressing the TRβ-320H (F67, CL) had serum T_4 and T_3 levels at the upper limit of normal, and in two others administration of 50 μg of T_3 for 7 d suppressed TSH and produced metabolic responses (15). These findings are not typical for subjects with RTH (1). Yet, R320H showed, by far, more severe impairment of transactivation function as well as dominant inhibition than any group B ARTmuts. Thus, it seems unlikely that mutations of the group B type would result in clinically detectable changes or significant alterations in parameters of thyroid function.

As for R316H, this mutation was originally found in a patient (G.H.) who presented with RTH that predominantly involved the pituitary (22). Although the same mutation was found in two other members of the family, their thyroid function tests were within the limits of normal, and failure to demonstrate pituitary resistance to thyroid hormone resulted in a dissociation between phenotype and genotype. Experimental data presented by Geffner et al. (22) showed that R316H had no dominant negative effect in a transient transfection assay using HeLa cells and MTV-IR-CAT as promoter. In the present report, however, we found that R316H is an efficient inhibitor of WT TRβ, though the magnitude of inhibition was reduced relative to the impairment in T_3 binding and transactivation function. These discrepant results, most likely due to the use of different TRE constructs and cell lines, do not mitigate the conclusion reached. In fact, both explain the relatively "mild form" of RTH in an unrelated family with the same mutation we identified recently (22a). In this family (F120), the three subjects harboring R316H all had mildly elevated free T_4 and T_3 serum levels. Thus, the experimental data correlate with the clinical and laboratory findings in family F120 but not with those of family GH.

R410X is the only ARTmut that lacked T_3 binding. However, this receptor was also completely devoid of dominant negative effect. This is not surprising since, as shown previously (56, 57), TRβ lacking 35 COOH-terminal amino acids cannot bind to TRE. R410X should not produce clinical or laboratory changes in heterozygous state, since complete deletion of the protein-coding region in TRβ in one allele is not associated with RTH (12). This mutation would manifest clinically in homozygotes and present a recessive pattern of inheritance.

Collectively, our results suggest that mutations expected to occur with the greatest frequency in the cold region of the ligand-binding domain of TRβ are not prone to detection. This is due to small or no alteration of T_3 binding and transactivation function and minimal or no dominant negative effect. While the latter was expected on the basis of the previously shown dimerization function of this region of the TRβ, the insensitivity to amino acid changes in terms of T_3 binding suggests that this region of the putative ligand-binding domain may not be involved in a direct interaction with T_3.

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