Thyroid Hormone Resistance Syndrome
Inhibition of Normal Receptor Function by Mutant Thyroid Hormone Receptors

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Abstract

Thyroid hormone (T₃) resistance is inherited in most cases in an autosomal dominant manner. The disorder is characterized by elevated free thyroid hormone levels and partial resistance to thyroid hormone at the cellular level. Distinct single amino acid substitutions in the ligand binding domain of the β form of the thyroid hormone receptor have been described in two kindreds with this disorder. We used transient expression assays to characterize the functional properties of these receptor mutants, one containing a Gly to Arg change at amino acid 340 (G340R) and the other a Pro to His change at amino acid 448 (P448H). A nine amino acid carboxy terminal deletion (Δ448-456), analogous to an alteration that occurs in v-erbA, was also studied for comparison with the mutations that occur in the T₃ resistance syndrome. None of the receptor mutants were able to mediate thyroid hormone dependent activation (TreTKCAT) or repression (TSHoCAT) of reporter genes when compared with the wild type receptor. In addition, the mutants inhibited the activity of normal α and β receptor isoforms when examined in coexpression assays. This activity, referred to as dominant negative inhibition, was manifest with respect to both the positively and negatively regulated reporter genes. Although mutant receptor binding to DNA was unaltered, ligand binding studies showed that the G340R and Δ448-456 mutants failed to bind T₃, whereas the P448H mutant bound hormone with reduced affinity (~ 10% of normal) compared to the wild type receptor. Consistent with this finding, the P448H mutant receptor was partially active at higher T₃ concentrations. Furthermore, the dominant negative inhibition elicited by the P448H receptor mutant at higher T₃ concentrations was reversed in the presence of high doses of T₃. These findings indicate that mutant β receptors in patients with thyroid hormone resistance have reduced affinity for T₃ and are functionally deficient, but impair the activity of normal receptors, thereby providing a mechanism for the dominant mode of inheritance in this disorder. (J. Clin. Invest. 1991. 87:1977–1984.) Key words: transcriptional regulation • erbA • thyroid-stimulating hormone • thyroid hormone resistance • thyroid hormone

Introduction

The syndrome of generalized thyroid hormone resistance (GTHR)¹ was first described by Refetoff et al. (1), and is characterized by elevated circulating levels of free thyroxine (T₄) and triiodothyronine (T₃) and inappropriate normal or increased levels of thyroid-stimulating hormone (TSH). Although patients with GTHR are relatively refractory to the metabolic effects of increased thyroid hormone, there is variability in the degree of resistance in different target tissues within an individual and the clinical phenotype varies in different kindreds (2). An autosomal recessive mode of inheritance was suggested in original GTHR kindred, but the disorder has been shown to segregate in an autosomal dominant manner in most families (1, 3).

Although both quantitative (4) and qualitative defects (5) in thyroid hormone binding to fibroblast nuclear receptors from GTHR patients have been described, these studies did not firmly establish a defect at the level of the thyroid hormone receptor. Recently, two thyroid hormone receptor genes, designated hTRα and hTRβ, have been identified, encoding highly homologous proteins with different tissue distributions (6, 7). Genetic analysis has now shown linkage between the GTHR syndrome and the β receptor gene locus in three kindreds (8, 9). These observations have been extended by sequencing β receptor cDNAs from two separate families with the disorder. In both cases, affected individuals have a point mutation in one allele of the β receptor gene together with a second normal allele, whereas their unaffected relatives have two normal alleles. In each kindred, the point mutation alters a single amino acid codon in the carboxy-terminal ligand binding domain of the receptor. In one kindred, the mutation is a Glycine to Arginine substitution at amino acid 340 (G340R) (10). In the other kindred, a Proline to Histidine substitution was identified at amino acid 448 (P448H) (9).

Although ligand binding studies have shown that these mutant proteins either fail to bind T₃ (10) or bind hormone with reduced affinity (11), their ability to modulate target gene expression has yet to be defined. Moreover, because the affected individuals also possess a second normal β receptor allele and presumably two normal α receptor alleles, the mechanism by which the mutant receptor causes resistance to thyroid hormone action is unclear. It has been hypothesized that the mutant receptor might block the activity of normal receptors (9, 10). In support of this concept, receptor homologues (v-erbA; splicing variant α2) that are unable to bind thyroid hormone

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1. Abbreviations used in this paper: ABCD, avidin-biotin complex DNA; CAT, chloramphenicol acetyltransferase; GTHR, generalized thyroid hormone resistance; Kᵣ, receptor affinity constant; RSV, Rous sarcoma virus; TRE, thyroid response element; nTRE, negative TRE; TREP, positive TRE.
have been shown to inhibit the action of the wild type receptors (12–14).

Transient expression assays provide a convenient model for examining the functional properties of thyroid hormone receptors. We have previously used a receptor deficient human cell line (JEG-3) for studies of the functional properties of human α and β receptor isoforms (15, 16). Both types of thyroid hormone receptor are capable of activating expression of a reporter gene (TreTKCAT) that contains a palindromic positive TRE (TREp) and repressing the activity of a reporter gene (TSHoCAT) that contains a negative thyroid response element (nTRE) (15, 16). Transcriptional stimulation and repression each require receptor activation by thyroid hormone.

In this study, we used this transient assay system to characterize the functional properties of the mutant receptors from two GTHR kindreds (G340R and P448H) as well as a carboxy-terminal truncated form of the β receptor (Δ448-456) that corresponds to the deletion in v-erbA (13). We demonstrate that in addition to being nonfunctional, the receptor mutants inhibit the action of their wild type receptor counterparts. This dominant negative action by the mutant receptors may explain the dominant mode of inheritance as well as resistance to hormone action.

Methods

Plasmid constructions and receptor mutagenesis. TSHoCAT contains 846 bp, of 5′ flanking sequence and 44 bp of exon 1 from the human TSH α subunit gene, linked to the gene encoding chloramphenicol acetyltransferase (CAT) (15). TreTKCAT (provided by G. Brent and D. Moore, Massachusetts General Hospital, Boston, MA) contains two copies of a palindromic TRE (5′ gatc TACGTCATGACCTGAGA-3′, linker sequences are shown in small case letters) inserted upstream of the thymidine kinase (TK) promoter and CAT gene (17).

For site directed mutagenesis (18), the human thyroid hormone β receptor cDNA was subcloned into M13mp18. The following oligonucleotides were used to direct second strand synthesis: G340R, 5′ CAGCTGAAAATTGGGCTCTGGGTGGTGTGTO 3′, P448H, 5′ ACA-GAACCCTCCTCCCCATTTGTTCTGGAAA 3′, β, Δ448-456, 5′-TGGCCCAAGACACTCCTCCCCATAGACTGACTGGATTTCTTCT-3′. Recombinant phage containing the desired mutations were identified by sequence analysis. Both mutant and wild type receptor cDNAs were then subcloned into a vector in which expression is driven by the Rous sarcoma virus (RSV) promoter (14). The mutations are illustrated schematically in Fig. 1 A.

Cell culture and transient expression assays. JEG-3 cells (HTB 36, American Type Culture Collection, Rockville, MD) were grown in Opti-Mem (BRL-Gibco Laboratories, Grand Island, NY) containing 2% (vol/vol) FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were trypsinized 18 h before transfection and plated into medium containing 2% FCS depleted of T3, by treatment with activated charcoal. Triplicate plates of cells were transfected with 5 μg reporter plasmid (TreTKCAT or TSHoCAT) together with 0.2–4 μg of wild type or mutant receptor expression vector with the addition of RSVLUC plasmid as necessary to maintain a constant amount of RSV-containing plasmid. Following a 16-h exposure to a calcium phosphate-DNA precipitate (19), fresh serum-stripped media were added with or without T3. After an additional 48 h, the cells were harvested and CAT enzyme activity was measured by quantitating the acetylation of [14C]chloramphenicol (20). The intraassay variation in CAT activity in triplicate transfections was typically < 10%. Absolute CAT assay values varied two to threefold in separate experiments, presumably reflecting differences in transfection efficiency. Representative experiments from a single group of transfections are therefore presented, but similar results were obtained in independent experiments.

Figure 1. Structures of wild type and mutant T3 receptors and reporter genes. (A) Schematic representation of human α and β receptor isoforms and β receptor mutants. Putative DNA (open boxes) and T3 binding domains are shown schematically. The positions of amino acid substitutions as well as a nine amino acid deletion in the β receptor are indicated. The T3 binding properties of these receptors are summarized at the right of the figure (+, normal binding; −, absent binding; ±, reduced binding affinity). (B) Reporter gene constructs used in transient expression assays. In TreTKCAT, the location of two copies of a positive TRE (TREp) is indicated by arrows. In TSHoCAT, the positions of two cAMP response elements (CREs) and the putative negative TRE (nTRE, hatched box) are diagrammed. The locations of the TATA box, transcriptional start site, and the CAT gene are also indicated for both constructs.

Receptor binding studies. Avidin-biotin complex DNA (ABC) binding assays were used to assess receptor binding to DNA as described previously (15). Both wild type and mutant β receptor cDNAs were subcloned into pGEM 7 (Promega Biotec, Madison, WI) for in vitro transcription and translation. The transcribed and capped mRNAs were used to program translation in a rabbit reticulocyte lysate system in the presence of [35S]radiolabelled methionine according to the protocol of the supplier (Promega). Aliquots of labeled protein were analyzed using SDS polyacrylamide gel electrophoresis to confirm receptor integrity and to demonstrate that comparable amounts of the different mutants were synthesized (data not shown). Double stranded TREp (16) or a control oligonucleotide (−72 to −32 bp in the human TSH α promoter) that does not bind to the thyroid receptor (15) were annealed, and the 3' recessed ends were filled in with biotin-11-dUTP (Enzo Biochem, Inc., New York, NY), dATP, dCTP, and dGTP using Klenow polymerase. Biotinylated DNA (500 fmol) was incubated with [35S]methionine labelled receptor (100,000 cpm of TCA precipitable protein) for 40 min at 24°C. The receptor-DNA complexes were precipitated with streptavidin-agarose beads (Bethesda Research Laboratories, Gaithersburg, MD) and quantitated by scintillation counting.

Receptor interactions with DNA were also analyzed using gel mobility shift assays in which nuclear receptor extracts were prepared from COS-1 cells (16). COS-1 cells (CRL 1650, American Type Culture Collection) were transferred to Opti-Mem with 2% charcoal stripped fetal bovine serum before transfection. Cells were transfected with CDM8 expression vectors (16) encoding human aI, b, or different site-directed mutants of the β receptor. After 36 h, cells were harvested and extracts
were prepared as described by Damm et al. (13). Binding reactions (50 μl) contained 32P-labelled TRESp (50 fmol) or a control oligonucleotide (−72 to −32 bp in the human TSH α promoter) incubated with transfected COS cell nuclear extract (7.5 μg protein) in binding buffer (20 mM Hepes pH 7.9, 120 mM KCl, 2 mM dithiothreitol, 0.1% NP-40, 100 μg/ml poly (dl-C)), and 100 nM T3). The DNA-protein mixture was incubated at room temperature for 60 min before electrophoresis through a 5% nondenaturing acrylamide gel.

For hormone binding assays, unlabelled in vitro translation products (5 μl) were incubated with 0.01 nM [3H]T3 and increasing amounts (0.001–0.4 nM) of T3 (21). The reactions were carried out at 4°C for 16 h in a final vol of 200 μl of binding buffer (10% (vol/vol) glycerol, 15 mM Tris pH 7.6, 50 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol). Nonsaturable binding was measured in the presence of a 1,000-fold excess of unlabeled T3. Bound and free hormone was separated by filtration (HAWP 2540; Millipore/Continental Systems, Bedford, MA) (22). Receptor affinity constants (Kd) were calculated using Scatchard plot analyses.

Results

Functional properties of mutant receptors. To assess the ability of wild type and mutant receptors to activate or repress transcription, TreTKCAT and TSHαCAT (Fig. 1 B) were used as reporter genes to measure transcriptional activation and repression, respectively, in thyroid hormone receptor deficient JEG-3 choriocarcinoma cells. Receptors and mutants were subcloned into an RSV expression vector. Using this system, maximal receptor activity is elicited using 100 ng of the receptor expression vector and 1 nM T3 (16). In these experiments, supramaximal doses (200 ng) of wild type or mutant receptor and T3 (5 nM) were used to ensure maximal receptor activity.

In the absence of cotransfected receptor, T3 treatment repressed TSHαCAT activity (20–50% suppression in different experiments) (Fig. 2 B). In other experiments, we have also observed minimal induction of TreTKCAT expression in the absence of transfected receptor (one to threefold, data not shown). These T3-mediated effects may reflect the presence of low amounts of endogenous receptor in these cells. Coexpression of wild type β receptor conferred a marked increase in T3 responsiveness. TreTKCAT expression was induced 18-fold and TSHαCAT was suppressed by 79% (Fig. 2 A). Under the same conditions, the mutant receptors exhibited markedly diminished activity. The β ΔAI48-456 mutant failed to mediate activation of TreTKCAT or repression of TSHαCAT. The GTHR receptor mutants (G340R and P448H) were also functionally deficient, but differed in their activities. Whereas G340R was essentially inactive (0.9-fold induction of TreTKCAT and 17% repression of TSHαCAT), P448H exhibited partial T3 responsiveness (4.2-fold induction of TreTKCAT and 35% repression of TSHαCAT).

Dominant negative action of mutant receptors. Having established the functional capabilities of the individual receptor mutants, their ability to modulate the activity of wild type α and β receptors was examined. Wild type β receptor (200 ng) was coexpressed with or without a 10-fold excess (2 μg) of each of the mutant receptors. Under these conditions, the receptor mutants markedly inhibited both positive and negative transcriptional responses mediated by the wild type receptor (Fig. 3). For example, T3-dependent activation of TreTKCAT was reduced from 73-fold with β receptor alone to 21-fold and 24-fold in the presence of coexpressed G340R or P448H, respectively. The ΔAI48-456 mutant was more potent and reduced activation to sixfold. A similar pattern of results was obtained with respect to negative regulation using TSHαCAT. Repression of TSHαCAT activity in the presence of wild type receptor alone (80%) was blunted to 16% by G340R, 50% by P448H, and repression was eliminated using ΔAI48-456.

Dose response studies were carried out to determine the amount of mutant receptor expression vector required to inhibit wild type receptor activity. Increasing concentrations of the G340R mutant receptor progressively reduced transcriptional activation of TreTKCAT from 22- to 7-fold (Fig. 4 A). Similarly, the degree of repression of TSHαCAT was reduced from 62% with wild type β receptor alone to 30% with a 10-fold excess of G340R (Fig. 4 B). In both cases, half-maximal inhibition of wild type receptor activity occurred using a four to fivefold excess of mutant receptor.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Functional properties of the wild type and mutant β receptors. Control plasmid (RSVLUC) or RSV expression vector (200 ng) encoding either wild type or mutant β receptors were transfected into JEG-3 cells with 5 μg of the indicated TreTKCAT or TSHαCAT reporter genes. Triplicate transfections were incubated in either the absence (dark bars) or presence (hatched bars) of 5 nM T3. CAT activity is expressed as percent chloramphenicol acetylation per milliliter of cell extract per hour. The data shown is the mean±SD of triplicate transfections. Relative stimulation or repression in the presence of T3 treatment is indicated above the bars. (A) Positive regulation of TreTKCAT by T3, (B) Negative regulation of TSHαCAT by T3.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effect of receptor mutants on positive and negative transcriptional regulation by wild type β receptor. JEG-3 cells were transfected with 5 μg of TreTKCAT or TSHαCAT together with either 2,200 ng of RSVLUC (Control), or 200 ng of β receptor and a 10-fold excess (2,000 ng) of RSVLUC or mutant β receptors. Triplicate transfections were incubated in either the absence or presence of 5 nM T3, and the results are presented as the relative activity of ΔAI48-456 to T3 treated plates. (A) Positive regulation of TreTKCAT by T3, (B) Negative regulation of TSHαCAT by T3.
The human α isoform of the thyroid hormone receptor is highly homologous (> 85%) to the β receptor in the central DNA binding and carboxy-terminal ligand binding domains (Fig. 1 A). Consonant with this observation, the α receptor isoform binds T3 and can activate and repress target reporter genes in JEG-3 cells in a manner similar to the β receptor (12, 16). To test the ability of the mutant receptors to inhibit wild type α receptor activity, experiments analogous to those with the β receptor were performed (Fig. 5). T3-dependent activation of TreTKCAT by the α receptor was reduced from 118-fold to 36- and 43-fold with the G340R and P448H mutants, respectively. The effect of the mutant receptors was less effective in their inhibition of T3 dependent suppression by α receptor. After treatment with T3, TSHαCAT was repressed by 84% in the presence of α receptor alone and the degree of repression was reduced to 61%, 64%, and 43% in the presence of excess G340R, P448H, and G448-456 mutants, respectively.

Ligand and DNA binding properties of the receptor mutants. As shown in the foregoing experiments, the mutant receptors exhibited somewhat different functional properties. When tested alone, the P448H mutant was capable of modulating target gene activity slightly, whereas G340R and Δ448-456 were completely inactive (Fig. 2). Likewise, when listed in order of potency as dominant negative inhibitors of wild type receptor activity (Fig. 3, 5), the rank order was Δ448-456 > G340R > P448H. One potential explanation for these results could involve differences amongst the receptors in their ligand binding properties. Therefore, each of the receptors was generated by in vitro transcription and translation to allow measurements of the affinity of T3 binding (Table I). The wild type receptor binding affinity (1.46 × 10^10 M^-1) was similar to values reported previously (21). The P448H mutant bound T3, but with an affinity that was reduced by a log order (1.52 × 10^9 M^-1). Binding to G340R and Δ448-456 mutants, if it occurs, was below detection in these assays.

Two different assays were employed to assess the ability of receptor mutants to bind to DNA. In the ABCD binding assay, [35S]methionine-labelled receptors were prepared by in vitro translation, and binding of labelled receptors to biotinylated DNA was measured after precipitation with streptavidin agarose beads (Fig. 6 A). Using a known receptor binding site (TREp), the binding of each of the ligand domain receptor mutants (G340R, P448H, Δ448-456) was comparable to that of the wild type β receptor, whereas a mutation in the DNA binding domain of the β receptor (C1225S (15)) resulted in negligible interaction with TREp.

Receptors were also expressed in COS-1 cells and used to assess DNA binding properties using gel mobility shift assays (Fig. 6 B) (16). In addition to providing an independent measurement of DNA binding, this assay has the potential advantage of detecting major alterations in mRNA or protein stability that might result from receptor mutations. As shown previously (16), untransfected COS cells contain little receptor binding activity (lane 3). However, after transfection with either wild type α or β receptors, distinct receptor-DNA complexes were formed using nuclear extracts and TREp (lanes 4 and 5), but not with control DNA that does not bind to receptor (15) (lanes 1 and 2). Expression and DNA binding of the G340R mutant (lane 6) and the Δ448-456 mutant (lane 7) were similar to the wild type β receptor. These assays indicate that the ligand binding domain mutations have not altered the ability of the receptors to interact with DNA.

T3 dose dependence of mutant receptor action. In light of its ability to bind T3 with reduced affinity, the properties of the P448H mutant receptor were examined at low and high T3 concentrations (Fig. 7). Activation and repression of reporter gene expression by wild type β receptor was similar in the presence of 0.75 nM or 100 nM T3 concentrations. The mutant
P448H was only weakly active (fourfold activation of TreTKCAT, 40% repression of TSHαCAT) at the low T₃ concentration, but became fully functional at the higher T₃ concentration. The apparent increase in the activity of the P448H mutant relative to the wild type β receptor at high T₃ doses was not observed in other experiments. Thus, although P448H binds T₃ with reduced affinity, it appears to function normally in the presence of saturating doses of hormone. As expected based upon the T₃ binding data, the reduced activity of mutants G340R and ΔA448-456 was unaffected by increasing the dose of T₃ (data not shown).

The dominant negative effects of the P448H receptor mutant were also dependent on T₃ concentration (Fig. 7). TreTKCAT activation by wild type β receptor was reduced from 25-fold to 4-fold by mutant P448H at 0.75 nM T₃, but TreTKCAT activation was not inhibited at 100 nM T₃. Similarly, repression of TSHαCAT activity was effectively blocked by mutant P448H at a low T₃ concentration, but full repression was retained at the high T₃ concentration.

Discussion

In this study, we have characterized the functional properties of mutant thyroid hormone receptors (G340R and P448H) that were identified previously in two separate kindreds with GTHR syndrome (9, 10). Several main conclusions can be drawn from these studies. First, the mutant receptors bind T₃ with greatly reduced affinity and their ability to transcriptionally activate or repress target genes in response to T₃ is therefore impaired. This observation is in keeping with previous studies showing T₃ receptor isoforms (e.g., v-erbA, α2) that cannot bind T₃ are transcriptionally inactive (12, 13, 16) and emphasizes the fact that transcriptional regulation is a ligand-dependent process. Second, the different receptor mutants have distinct functional properties. Whereas G340R and ΔA448-456 exhibit no T₃ binding and are completely inactive in transfection assays, P448H binds T₃ with reduced affinity, and consequently is partially active in response to T₃. Third, we find that the mutant receptors inhibit the transcriptional activity of the wild type thyroid hormone receptor. Notably, the inhibition by mutant receptors applies to the activity of both β and α receptors and is manifest using both positively and negatively regulated target genes.

Two major mechanisms might be considered to explain the fact that GTHR can be inherited in an autosomal dominant manner. Given that the mutant receptors are transcriptionally inactive, one possibility is that the amount of functional receptor is reduced as a consequence of having the mutation occur in one of two autosomal alleles. Alternatively, it is possible that the mutant receptor in some manner interferes with the function of normal receptors, thereby accounting for its phenotypic expression in the heterozygous state. The receptor deficiency mechanism assumes that the quantity of functional receptor within the cell is a limiting step for hormone action and that the normal allele is not upregulated to compensate for the mutant allele. There is currently no evidence that excludes this mechanism. However, the observation that mutant receptors can block the transcriptional regulation of two different reporter genes supports an interference mechanism as the basis for resistance to thyroid hormone action in heterozygotes.

Given the apparent complexity of transcriptional regulation by thyroid hormone receptors, it is useful to consider potential cellular mechanisms by which mutant receptors could inhibit wild type receptors. One mechanism could involve the formation of nonfunctional heterodimers between wild type and mutant receptor proteins (Fig. 8, model 1). This type of
mechanism has been documented with other DNA binding transcription factors. For example, in the CREB/ATF family of transcription factors, homodimers and heterodimers form via a conserved leucine zipper motif (23) and mutant CREB proteins function in a dominant negative manner by dimerizing with the wild type protein to inhibit transcriptional capability (24). Another such example is provided by the Id protein which inhibits the transcriptional activity of the muscle differentiation factor MyoD by forming heterodimers via a shared helix-loop-helix (25). In the case of thyroid hormone receptors, deletion mutants of the chicken α receptor repress wild type receptor function and this inhibitory property has been localized to the carboxy-terminal domain that may contain a dimerization interface (14, 26). In support of this concept, the thyroid hormone receptor has recently been shown to form heterodimers with the retinoic acid receptor via a shared structural motif within the carboxy-terminal region (27). Both of the GTHR receptor mutations occur outside of this putative dimerization domain, raising the possibility that they could still form dimers. Further structure-function analyses within this region will be required to precisely delineate the receptor sequences that are required for receptor–receptor interactions.

A second mechanism for dominant negative activity would be for the transcriptionally defective mutants to compete with the wild type receptor for binding to a common DNA target sequence, which in this case is the TRE (Fig. 8, model 2). The finding that a mutation in the DNA binding domain of the viral oncogene, v-erbA, abolishes its dominant negative properties, lends support to this type of mechanism (13). The introduction of DNA binding domain mutations into the GTHR receptors may provide further insight regarding the contribution of this mechanism to their dominant negative activity. It is interesting to note that in another receptor resistance syndrome (hereditary resistance to vitamin D), the disorder is inherited in an autosomal recessive manner and heterozygotes are phenotypically normal (28). In this instance, the mutations occur within the DNA binding domain of the vitamin D receptor, consistent with the hypothesis that binding to DNA may be required for receptors to exert inhibitory activity in the heterozygous state.

The mutant receptors could also inhibit the action of normal receptors by sequestering a shared accessory factor that is necessary for wild type receptor modulation of gene transcription (Fig. 8, model 3). This property, sometimes referred to as "squelching" (29), has been postulated to explain transcriptional interference between coexpressed estrogen and progesterone receptors (30), and recent analyses of transcriptional regulation by the adenovirus E1a protein also support this concept (31). Because inhibition by the GTHR and A448-456 mutants occurs with relatively low doses of coexpressed mutant receptors, we do not favor such a mechanism for these mutants, but it cannot be excluded at this point. These three potential pathways for transcriptional interference by the mutant receptors are not mutually exclusive and inhibition may well result from a combination of several different mechanisms.

In many respects, the dominant negative properties that we have observed with the GTHR receptor mutants are analogous to the findings with other transcriptionally inactive thyroid hormone receptor variants (12, 13, 16). For example, both the v-erbA oncogene and the α2 isoform have been shown to act as dominant negative inhibitors when coexpressed with the wild type receptor in transient expression assays. The GTHR mutants, v-erbA, and α2 receptors share in common the fact that they contain sequence alterations in the extreme carboxy terminus of the receptor. Although these alterations eliminate or greatly reduce hormone binding in each of these mutants, it may be premature to conclude that this is the only modification in receptor function that is required for dominant negative activity. For example, the carboxy-terminal nine amino acid deletion that occurs in v-erbA has been proposed to serve as a transactivation domain (32). Thus, its deletion could potentially eliminate both T3 binding and the potential for transactivation. As suggested in Fig. 8, the activity of heterodimers formed between v-erbA and a normal cellular receptor could potentially be influenced by the presence or absence of a transactivation domain within v-erbA. This feature might account for the somewhat greater inhibition that is observed with the v-erbA homologue (A448-456) than with the GTHR mutants (G340R, P448H) (Figs. 3 and 5), or with the α2 isoform (16).

If an interference mechanism is the basis for thyroid hormone resistance in the heterozygous state, then in the simplest case it might be proposed that the normal and mutant alleles would be expressed equally resulting in a 1:1 ratio of normal and mutant receptor. The fact that mutant and wild type sequences comprise a similar number of isolated cDNA clones (10) provides some data to support this assumption. However, several lines of evidence suggest that the situation in vivo may be complex. For example, T3 can regulate the expression of its own receptors (33, 34) and the impact of thyroid hormone resistance on this process is currently unknown. Furthermore, the levels of expressed α and β receptors varies widely in different tissues (34, 35) and a number of receptor splicing variants
have been recognized for both forms of receptor (36–38). Thus, if the mutation occurs in one of the β receptor genes, the degree of resistance may reflect the composition of receptor isoforms within a given cell as well as compensatory responses of the various receptor isoforms to the T3 resistant state. Future studies of receptor expression and regulation in vivo may help to clarify the relative proportions of different receptor isoforms.

We examined the issue of the stoichiometry of mutant recep-
tor inhibition in vitro by altering the ratio of mutant to wild type receptors in transient expression assays (Fig. 4). Although some degree of dominant negative activity was apparent at low mutant/wild type receptor ratios (1:1 or 2:1), it was somewhat surprising that 50% inhibition required at least a fourfold ex-
cess of mutant receptor. The basis for the requirement for ex-
cess mutant receptor and its relevance to clinical thyroid hor-
mones are unclear. It is possible that clinical measurements of resistance (e.g., elevated thyroid hormone levels) and more sensitive indicators of receptor inhibition than our in vitro assays of transient gene expression. A more likely basis for the requirement for excess mutant receptor may involve the artificial nature of the transient expression assay. Although the predicted ratios of expressed receptor are likely to correlate with the amounts of transfected plasmid, we do not have a quantitative measure of the expressed receptor proteins. Based upon their levels of expression in transfected COS cells (Fig. 6 B), there is some evidence that the stabilities of the mutant and wild type receptor mRNAs and proteins are similar in trans-
fected cells. It is possible that quantitative differences in domi-
nant negative activity would be found under different condi-
tions such as performing the experiments at a lower or higher level of expressed reporter gene or using a different level of wild type receptor. In addition, the current studies have been performed in only a single receptor deficient cell line (JEG-3 cells). A number of recent reports indicate that T3, T3 receptors interact with other cellular proteins (27, 39–41) and the levels and com-
positions of such factors may alter dominant negative regula-
tion in a cell type specific manner. One must also consider that the inhibitory effects of the mutant receptors may be depend-
ent upon the target gene for thyroid hormone receptor action. For example, coexpressed thyroid hormones and retinoic acid receptors act cooperatively to induce the expression of a target gene containing a palindromic TRE from the myosin heavy chain gene (27). Because of these issues, it is apparent that one should interpret transient receptor assays of receptor function cautiously, particularly with respect to quantitative effects. De-
spite these reservations, this technique appears to provide a valuable in vitro assay for the transcriptional activity of recep-
tor mutants and to serve as an index of dominant negative activity.

The observation that different kindreds with GTHR dis-
distinct clinical features is a particularly intriguing aspect of this disorder (2, 3, 9). We have shown that the receptor mutants from two kindreds differ in their functional properties. At a low T3 concentration, the properties of P448H and G340R are essentially indistinguishable in vitro. However, at a T3 concentration which saturates P448H, this mutant is trans-
criptional active and loses its dominant negative activity. How-
ever, we note that individuals with the P448H mutation (9) and the G340R mutation (10) have similar circulating thyroid hor-
mones and the clinical features of resistance are different, but not necessarily less severe, in individuals with the P448H mutation. Thus, it is unclear at this stage whether differences in ligand affinity or some other aspect of mutant receptor func-
tion account for phenotypic differences in this disorder. To better understand the clinical features that result from thyroid hormone resistance, it will be necessary to acquire more informa-
tion about the in vivo distribution, regulation, and mechan-
isms of action of normal and mutant receptors. Recent stud-
ies (9, 42) suggest that a number of different mutations may exist in other kindreds with GTHR. Analyses of their func-
tional properties will be of interest to further elucidate the pathophysiology of this disorder.

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