A role for helix 3 of the TR β ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone

Trevor N.Collingwood, Richard Wagner¹, Clare H.Matthews, Rory J.Clifton-Bligh, Mark Gurnell, Odelia Rajanayagam, Maura Agostini, Robert J.Fletterick¹, Paolo Beck-Peccoz², Walter Reinhardt³, Gerhard Binder⁴, Michael B.Ranke⁴, Ad Hermus⁵, Rolf D.Hesch⁶, John Lazarus⁷, Paul Newrick⁸, Vernon Parfitt⁹, Peter Raggatt¹⁰, Francis de Zegher¹¹ and V.Krishna K.Chatterjee¹²

Department of Medicine, University of Cambridge, Level 5, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, UK, ¹Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA, ²Istituto Clinico Humanitas, Milan, Italy, ³Abteilung für Endokrinologie, Universitätsklinikum Essen, Germany ⁴Kinderklinik, Universität Tübingen, Germany, ⁵Department of Endocrinology, University Hospital Nijmegen, The Netherlands, ⁶Seestrasse 1, Konstanz, Germany, ⁷Department of Medicine, University of Wales College of Medicine, Cardiff, ⁸Department of Medicine, Kidderminster General Hospital, Kidderminster, ⁹Department of Endocrinology, Royal South Hants Hospital, Southampton, ¹⁰Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge, UK and ¹¹Department of Paediatrics, University of Leuven, Leuven, Belgium

¹²Corresponding author

Resistance to thyroid hormone (RTH) has hitherto been associated with thyroid hormone β receptor (TR β) mutations which cluster in two regions ($\alpha\alpha$ 310–353 and $\alpha\alpha$ 429–461) of the hormone-binding domain and closely approximate the ligand-binding cavity. Here, we describe a third cluster of RTH mutations extending from $\alpha\alpha$ 234–282 which constitute a third boundary of the ligand pocket. One mutant, T277A, exhibits impaired transactivation which is disproportionate to its mildly reduced ligand affinity (K_d). T3-dependent recruitment of coactivators (SRC-1, ACTR) by mutant receptor–RXR heterodimers was reduced in comparison with wild-type. Cotransfection of SRC-1 restored transactivation by T277A. In the TR β crystal structure this helix 3 residue is surface-exposed and is in close proximity to residues L454 and E457 in helix 12 which are known to be critical for coactivator interaction, suggesting that they all constitute part of a receptor–coactivator interface. The transcriptional function of other mutants (A234T, R243W/Q, A268D, Δ 276I, A279V, R282S) in this cluster correlated with their reduced K_d and they inhibited wild-type TR β action in a dominant negative manner. DNA binding, heterodimerization and corepressor recruitment were preserved in all mutants, signifying the importance of these attributes for dominant negative activity and correlating with the absence of natural mutations in regions bordering the third cluster which mediate these functions.

Keywords: coactivator/dominant negative/resistance to thyroid hormone/transactivation/thyroid receptors

Introduction

In keeping with other members of the nuclear receptor family, TR β is a ligand-inducible transcription factor with a modular structure comprised of a central DNA-binding (C) domain flanked by a C-terminal (D/E/F) region that encompasses ligand binding, dimerization and hormone-dependent transactivation functions and an N-terminal (A/B) region implicated in ligand-independent transactivation (Mangelsdorf and Evans, 1995). In many target genes, TR interacts with regulatory DNA response elements typically consisting of hexanucleotide motifs oriented as direct, inverted or everted repeats. TR functions primarily as a heterodimer with the retinoid X receptor (RXR) but can function as a homodimer in some contexts (Lazar, 1993). In the absence of ligand, TR actively represses basal gene transcription by recruiting a complex containing corepressor proteins (SMRT or NCoR) (Chen *et al.*, 1995; Hörlein *et al.*, 1995) associated with a histone deacetylase (HDAC) via an intermediary factor (Sin3) (Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Receptor occupancy by T3 results in dissociation of this corepressor complex and derepression, followed by recruitment of coactivators and associated cofactors (e.g. RIP140, SRC-1, TIF-2, CBP, ACTR, p/CAF) exhibiting histone acetyltransferase activity, as well as other associated factors, subsequently leading to transcriptional activation (Cavaillès *et al.*, 1995; Chen *et al.*, 1997; Oñate *et al.*, 1995; Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Voegel *et al.*, 1996). Residues at the extreme C-terminus in many nuclear receptors have been shown to be critical for coactivator recruitment, and recent crystallographic studies indicate that these residues lie within a solvent-exposed amphipathic α -helix which may undergo a conformational change in response to ligand (Renaud *et al.*, 1995). However, the role of other regions of the receptor which might play a part in this recruitment remains to be determined.

Over 70 different natural TR β mutations have been identified in the syndrome of resistance to thyroid hormone (RTH), the hallmark feature of which is elevated circulating free thyroid hormones (thyroxine, T4 and triiodothyronine T3) together with non-suppressed levels of thyroid stimulating hormone (TSH). These mutations all occur within the C-terminal ligand-binding domain (LBD) (Refetoff *et al.*, 1993) and characterization of their properties has made a valuable contribution to the understanding of structure–function relationships in TR. With a few exceptions, a consistent abnormality with natural receptor mutants is a significant reduction in their ligand-binding

affinity resulting in impaired hormone-dependent transcriptional activity (Meier *et al.*, 1992; Collingwood *et al.*, 1994). In addition, the mutant receptors inhibit the action of their wild-type counterparts in a dominant negative manner (Sakurai *et al.*, 1990; Chatterjee *et al.*, 1991), a property which is central to the pathogenesis of the RTH phenotype and consistent with their dominant mode of inheritance. This inhibitory effect is dependent on their ability to bind to DNA (Nagaya *et al.*, 1992), heterodimerize with RXR (Nagaya and Jameson, 1993; Collingwood *et al.*, 1994) and recruit corepressor (Yoh *et al.*, 1997). These findings correlate with the observation that no natural RTH mutations have been identified in the DNA-binding domain (DBD) of TR β and, moreover, that the majority of mutations occur in two clusters ($\alpha\alpha$ 310–353; $\alpha\alpha$ 429–461) wherein the intervening region contains a putative dimerization interface implicated in receptor interaction with RXR (Forman and Samuels, 1990). We have also described RTH mutants that interact aberrantly with transcriptional cofactors: a natural mutant, L454V, with relatively preserved ligand- and DNA-binding and dimerization functions (Collingwood *et al.*, 1997) was impaired transcriptionally as a result of attenuated interaction with putative coactivators (RIP140, SRC-1); another mutant, R383H, was predominantly impaired for negative transcriptional regulation and hormone-dependent corepressor release (Clifton-Bligh *et al.*, 1998).

Here, we report a mutant (T277A) within a third natural mutation cluster ($\alpha\alpha$ 234–282), which is impaired transcriptionally with reduced coactivator binding. We suggest that helix 3 of the LBD which encompasses this residue, together with helix 12, may constitute part of a wider receptor–coactivator interface. Other natural mutants in this cluster show variably impaired ligand binding and transcriptional function and inhibit wild-type TR action in a dominant negative manner, but retain DNA- and corepressor-binding and heterodimerization functions. These observations may explain the absence of naturally occurring mutations in receptor domains (α -helical extension of the DBD, signature motif, CoR box) bordering this third cluster.

Results

Genotype and clinical phenotype of RTH cases

Thyroid function tests and receptor gene abnormalities in affected members of 14 different RTH families are shown in Table I. Most individuals exhibited biochemical abnormalities characteristic of RTH with elevated serum-free T4 and T3 levels together with an inappropriately normal TSH. Three patients (IV, IX, XIII.I) had undergone a previous subtotal thyroidectomy, disrupting the pituitary–thyroid feedback axis, resulting in variably elevated TSH levels with raised (IV, IX) or normal (XIII.I) serum-free T4 concentrations. Sequencing of the TR β gene indicated that each affected individual was heterozygous for either a single nucleotide substitution corresponding to a missense mutation or, in one case, a three nucleotide in-frame codon deletion (Δ 276I). Five novel mutations (A268D, Δ 276I, T277A, A279V, R282S) have not been described previously in RTH. In addition, we have identified and characterized more extensively mutations (V264D, A234T, R243W, R243Q) which have been documented as case reports

(Behr and Loos, 1992; Adams *et al.*, 1994; Onigata *et al.*, 1995; Pohlenz *et al.*, 1996). In one instance (II), a de novo R243W mutation exists in the proband but not in either parent, supporting the hypothesis that these identical mutations have arisen independently in unrelated families.

RTH mutant receptors show variably impaired transactivation function

In keeping with their location in the TR LBD, the initial characterization of mutant receptors consisted of measurement of the ligand-binding affinities (K_a) of *in vitro* synthesized proteins. All showed variably reduced T3 binding, with a few (A279V, A268D, Δ 276I) having affinities too low to quantitate (Table I). To evaluate their transcriptional properties, wild-type or mutant receptors were cotransfected with reporter genes containing one of three different types of thyroid response element (TRE) into JEG-3 cells with increasing concentrations of T3. All mutants were impaired transcriptionally on the direct repeat (MAL-tkLUC), everted repeat (F2-tkLUC) and palindromic (PAL-tkLUC) TREs (Figure 1A–C) and, as expected, those mutants with greatly reduced ligand binding (A279V, A268D, Δ 276I) showed the greatest transcriptional impairment, demonstrating submaximal responses even in the presence of saturating concentrations (1000 nM) of T3. For most mutants with measurable ligand-binding affinities (A234T, R243Q, R243W, R282S), the extent of their transcriptional impairment when compared with wild-type receptor correlated negatively with their K_a (Figure 1D–F). However, a single mutant, T277A, exhibited submaximal transactivation on MAL-tkLUC and F2-tkLUC, despite saturating T3 concentrations (Figure 1A and B), but not on PAL-tkLUC (Figure 1C). Furthermore, the T3 concentration required for T277A to attain 50% wild-type receptor activity on the direct repeat and everted response elements was markedly discordant with its K_a (Figure 1D and E). This pattern of transcriptional impairment suggested that the T277A mutation might disrupt some function of the receptor other than ligand binding.

Reduced transactivation by the T277A mutant is due to impaired coactivator recruitment

The attenuated maximal transactivation attained by the T277A mutant on MAL-tkLUC and F2-tkLUC was analogous to the activation profiles on these reporters of a number of mutants of a proline residue (P453A/H/S/T) which precedes the C-terminal amphipathic α -helix of TR (Collingwood *et al.*, 1994). Subsequent studies have shown that the P453S mutant receptor impairs hormone-dependent corepressor release to a greater extent than predicted from its reduced ligand-binding affinity (Yoh *et al.*, 1997). Hence, we examined whether the impaired transcriptional function of T277A might also be accounted for by abnormal corepressor release. Wild-type TR dissociated from GST–SMRT in response to T3 with an EC₅₀ of 4 nM (Figure 2). In contrast, the T277A mutant showed impaired ligand-dependent corepressor release such that its EC₅₀ was 20 nM (Figure 2). However, the R282S mutant receptor, which has an almost identical K_a in comparison with T277A (Table I), yet transactivates fully (Figure 1A–C), showed a similar alteration in corepressor release (Figure 2). We conclude that the delayed corepressor

Fig. 1. (A–C) T3-dependent transcriptional activation by mutant thyroid hormone receptors on different configurations of TRE. JEG-3 cells were cotransfected with wild-type or mutant TR β expression plasmids, along with the specified reporter construct (PAL-tkLUC, MAL-tkLUC or F2-tkLUC) an internal control plasmid (BOS- β gal), and incubated in the presence of increasing amounts of T3. Hormone-dependent activation was normalized against the internal control and expressed as the percentage of maximum wild-type receptor response. These data are the mean of at least three separate experiments performed in triplicate. The error for each point is <10%. (D–F) Correlation of transcriptional function of wild-type and mutant receptors with measurable hormone binding affinities (boxes: WT, A234T, R243W, R243Q, R282S; cross: T277A) with their respective K_a on direct repeat, everted and palindromic response elements. In each case, the concentration of T3 required to attain 50% of the wild-type maximal response [denoted as EC50 (wild-type)] is plotted against log K_a .

Table I. Biochemical and genetic data from 14 RTH families

Kindred	Age/sex	FT4	FT3	TSH	Nucleotide change	Codon change ^a	K_a ^b mean $\pm$ SEM
I	13 F	26	13.8	3.0	985 GCC to ACC	A234T	0.66 (0.04)
I.I	45 F	28	12.6	2.0	985 GCC to ACC	A234T	
II ^c	7 M	27	12.0	11	1012 CGG to TGG	R243W	0.19 (0.03)
III	27 F	68	16.0	3.5	1012 CGG to TGG	R243W	
III.I	67 M	28	9.5	0.4		R243W	
IV ^c	40 M	35	11.0	2.7	1012 CGG to TGG	R243W	
IV.I	4 M	38	15.0	4.5	1012 CGG to TGG	R243W	
V	70 F	28	9.5	0.5	1013 CGG to CAG	R243Q	0.25 (0.03)
VI	13 F	40	11.1	1.5	1013 CGG to CAG	R243Q	
VI.I	47 M	49	9.7	0.9	1013 CGG to CAG	R243Q	
VII	56 F	34	10.0	2.1	1013 CGG to CAG	R243Q	
VIII	20 M	29	15.0	2.5	1076 GTT to GAT	V264D	
IX ^c	40 M	31	16.6	10.4	1088 GCC to GAC	A268D	<0.2
X	20 M	55	20.6	2.1	1088 GCC to GAC	A268D	
XI ^d	7 F	32		2.1	1111-3 Δ ATC	Δ 276I	BND
XII	31 F	27	10.3	1.6	1114 ACA to GCA	T277A	0.46 (0.04)
XIII	26 F	27	9.7	1.5	1121 GCA to GTA	A279V	<0.2
XIII.I ^c	30 F	17	7.4	14.9	1121 GCA to GTA	A279V	
XIV	31 F	22	8.2	3.4	1131 AGA to AGT	R282S	0.47 (0.04)
XIV.I	37 M	29	13.5	3.3	1131 AGA to AGT	R282S	
XIV.II	34 F	24	11.1	1.4	1131 AGA to AGT	R282S	
XIV.III	60 M	20	10.0	3.4	1131 AGA to AGT	R282S	
XIV.IV	10 F	33	15.4	2.9	1131 AGA to AGT	R282S	
Normal range		9.0–20 pM	3.0–7.5 pM	0.4–4.0 mU/L	1.48×10^{10} (0.09) M ⁻¹		

^aCodon nomenclature based on a predicted protein sequence of 1–461 residues (Beck-Peccoz *et al.*, 1994); ^bBND, binding not detected; ^csubtotal thyroidectomy; no thyroid hormone replacement therapy; ^dtreated with TRIAC (interferes with FT3 assay).

Fig. 2. Using a pull-down assay, bacterially expressed GST-C-SMRT was incubated with equal amounts of *in vitro* translated wild-type or mutant (T277A, R282S) TR β in the presence of increasing amounts of T3. Wild-type TR β (—○—), T277A mutant (●, hatched line) and R282S mutant (●, dashed line) dissociation was quantitated using a phosphorimager. Each point represents the percentage of receptor protein bound to SMRT relative to the maximum bound by that receptor. Data represent the mean $\pm$ SE of four separate experiments.

release profiles exhibited by these two mutant receptors are in keeping with their reduced K_a and do not account for the diminished transcriptional function shown by T277A.

We have shown previously that another RTH mutant with markedly impaired transactivation, L454V, exhibited reduced coactivator recruitment *in vitro* (Collingwood *et al.*, 1997). Accordingly, we next examined the ability of wild-type or T277A mutant receptors to recruit coactivator proteins when bound to DNA as a heterodimer with RXR. Wild-type TR–RXR complexes, when bound to the direct repeat TRE, recruited glutathione *S*-transferase (GST) fusion proteins containing the central receptor-interacting domains of either SRC-1 (GST–SRC'; residues 570–780) or ACTR (GST–ACTR'; residues 621–821) in a T3-dependent manner (Figure 3A and B). In contrast, recruitment of GST–SRC' by T277A–RXR heterodimers on this response element was diminished (Figure 3A), with maximal coactivator recruitment reduced to 60% that of wild-type TR when quantitated (data not shown). Hormone-dependent recruitment of GST–ACTR' by T277A was also markedly impaired in comparison with wild-type (Figure 3B). These results suggest that reduced coactivator recruitment by T277A *in vitro* might account for its impaired transactivation *in vivo*. Indeed, we hypothesized that if impaired recruitment of endogenous coactivators was limiting the maximal transcriptional response of the T277A mutant, then overexpression of such factors might alleviate this defect. Accordingly, wild-type or T277A mutant receptors were cotransfected with Mal-*tkLUC* and increasing amounts (0–100 ng) of SRC-1 expression vector (Figure 4). Whereas the basal promoter (RSV) activity in mock-transfected cells or hormone-dependent activation by wild-type receptor were not affected significantly by co-expression of SRC-1, the transcriptional response of the T277A mutant was enhanced to wild-type levels.

Dominant negative activity of mutant receptors in the third cluster

It has been suggested that mutant receptors inhibit the action of wild-type receptor in a dominant negative manner to manifest the RTH phenotype (Collingwood *et al.*, 1994;

Fig. 3. Differential recruitment of coactivators by wild-type TR β and T277A when bound to DNA as a heterodimer with RXR. Using an electrophoretic mobility supershift assay, *in vitro* translated TR (wild-type or mutant T277A) and RXR were coincubated with the malic enzyme DR+4 TRE, along with either bacterially expressed GST–SRC' (2.4 μ g) (A) or GST–ACTR' (0.6 μ g) (B) in the presence of increasing concentrations of T3. Complexes were resolved by native PAGE.

Fig. 4. Restoration of T277A activity by overexpression of SRC-1. JEG-3 cells were cotransfected with 50 ng of RSV-TR wild-type or T277A mutant or empty vector, along with increasing amounts of pSG5-SRC1, as well as 500 ng of reporter MAL-*TKLUC* and 100 ng of the internal control Bos β gal. Empty pSG5 vector was added to maintain the amount of total DNA transfected. Cells were incubated in the presence or absence of 1 μ M T3. Data are the mean $\pm$ SE of three separate experiments performed in triplicate and activity is expressed relative to wild-type T3-dependent activation at 100 ng SRC-1.

Fig. 5. Dominant negative inhibition of wild-type receptor activity by mutant receptors on different TRE configurations. JEG-3 cells were cotransfected with equal amounts (100 ng) of wild-type plus each mutant TR β expression plasmid, along with the specified reporter constructs (A) PAL-tkLUC, (B) F2-tkLUC or (C) MAL-tkLUC and an internal control plasmid (BOS- β gal). The transfected cells were incubated in the presence of either low (1 nM; solid bars) or high (1000 nM; white bars) concentrations of T3. Hormone-dependent activation was normalized against the internal control and expressed as the percentage of maximum wild-type receptor response at each concentration of T3. These data are the mean $\pm$ SE of at least three separate experiments performed in triplicate.

Yen and Chin, 1994). We therefore examined the dominant negative potency of RTH mutants by co-expressing equal amounts of each with wild-type TR, again using three reporter genes containing different configurations of TRE in the presence of low (1 nM) or high (1000 nM) levels of T3 (Figure 5A–C). At 1 nM T3 all mutants showed marked dominant negative inhibition of wild-type receptor function on all three TREs. Reversal of this inhibition for some mutants (R243Q, R243W, R282S) at higher (1000 nM) T3 concentrations was in keeping with their ability to attain wild-type transactivation levels with saturating levels of ligand. Other mutants (A268D, Δ 276I, A279V, T277A, A234T) retained dominant negative activity at high T3 levels, presumably as a consequence of marked impairment either in hormone binding and/or transactivation function.

DNA binding and heterodimerization with RXR is preserved for all RTH mutants

One proposed mechanism of dominant negative inhibition by mutant receptors suggests that they form transcriptionally defective heterodimers with RXR which bind to DNA and compete with wild-type TR–RXR complexes at target gene TREs (Yen and Chin, 1994). Accordingly, we used an electrophoretic mobility shift assay to examine these properties for each RTH mutant using the three different TRE configurations utilized previously to test their transcriptional function (Figure 6A–C). All the RTH mutants bound DNA and heterodimerized with RXR comparably with wild-type receptor on palindromic (PAL), everted repeat (F2) or direct repeat (malic enzyme) TREs. In addition, both wild-type and mutant receptors bound DNA as homodimers with the everted repeat TRE (Figure 6B). In response to ligand (10 nM T3) the wild-type TR homodimer dissociated from DNA as described previously (Collingwood *et al.*, 1994), whereas the variable dissociation of the RTH mutant homodimers was concordant with their reduced ligand binding.

Mutant receptors recruit corepressor and repress basal transcription

Recent studies have suggested that the ability to silence basal transcription and recruit corepressor are important

Fig. 6. DNA binding, T3-dependent homodimer dissociation and dimerization of wild-type and mutant receptors with RXR on different configurations of TRE. In an electrophoretic mobility shift assay equal amounts of *in vitro* translated TR β mutants were incubated with RXR, along with 32 P-labelled DNA representing three different configurations of TRE. (A) Direct repeat element from the malic enzyme gene, (B) everted repeat element (F2) from the chicken lysozyme and (C) palindromic element derived from the rat growth hormone gene, in either the presence (+) or absence (–) of 10 nM T3. Complexes were resolved by native PAGE.

Fig. 7. Repression of basal promoter activity by TR β mutants and recruitment and T3-dependent release of corepressor SMRT. **(A)** Plasmids expressing fusion proteins containing the DNA-binding domain of Gal4 linked to the ligand-binding domain of TR β wild-type and mutants were cotransfected into 293 cells along with a reporter plasmid containing the Gal4 response element (UAS-TKLUC) and an internal control plasmid, BOS- β gal. Reporter activity was normalized against the internal control and is expressed relative to reporter activity in the presence of the Gal4 DNA-binding domain only (pSG424), which has a nominal value of 1. Data are the mean $\pm$ SE of at least three individual experiments performed in triplicate. **(B)** Using a pull-down assay, bacterially expressed GST-C-SMRT was incubated with equal amounts of *in vitro* translated TR β mutants in the presence (+) or absence (-) of 1 μ M T3. 'Input' represents 10% of the amount of receptor used in each reaction. We have shown previously that TR is not recruited to GST alone (Clifton-Bligh *et al.*, 1998).

attributes of RTH mutants and that the introduction of additional artificial mutations which abolish corepressor binding abrogates their dominant negative activity (Yoh *et al.*, 1997). We therefore examined the ability of each mutant to repress basal transcription by co-expressing fusions of wild-type or mutant TR LBDs linked to the DNA-binding domain (DBD) of Gal4 with a reporter gene containing Gal4-binding sites. In the absence of ligand, the wild-type Gal4-TR fusion repressed basal transcription to ~15% of that observed with the Gal4 DBD alone. In comparison, each RTH mutant repressed to a similar degree, suggesting that corepressor recruitment was preserved (Figure 7A). In contrast, two artificial mutants (P214R, L428R) exhibited markedly impaired silencing function. The P214R substitution is homologous to a natural mutation which reverses oncogenesis and dominant negative activity of *v-erbA* (Damm and Evans, 1993). We and others have previously shown that the L428R mutation abolishes both receptor dominant negative activity and heterodimerization with RXR (Nagaya and Jameson, 1993; Collingwood *et al.*, 1994), and recently the homologous TR α mutant (L374R) has been shown to lack corepressor interaction (Zhang *et al.*, 1997).

From these observations, we hypothesized that the interaction of each mutant receptor with corepressor is retained, and we tested this by studying the interaction between bacterially expressed GST-SMRT fusion protein and 35 S-labelled, *in vitro* translated mutant receptors. In the absence of ligand, all RTH mutants bound SMRT comparably with wild-type receptor (Figure 7B). Following the addition of saturating concentrations (1 μ M) of T3, most mutants dissociated from corepressor although, as expected, those with markedly reduced ligand-binding affinity (V264D, Δ 276I, A268D) exhibited impaired hormone-dependent corepressor release.

Discussion

A third cluster of mutations in resistance to thyroid hormone

The association of dominantly inherited RTH with the TR β gene locus has led to the identification of an expanding repertoire of mutations in the receptor-hormone binding domain. Rather than being uniformly distributed, they cluster mainly in two areas ($\alpha\alpha$ 310–353; $\alpha\alpha$ 429–461) within the LBD (Parrilla *et al.*, 1991; Refetoff *et al.*, 1993). There have been isolated reports of amino acid changes involving three codons outside these two clusters: our previous description of a valine to aspartic acid substitution at codon 454 (V264D; Adams *et al.*, 1994; Collingwood *et al.*, 1994); an alanine to threonine substitution at codon 234 (A234T; Behr and Loos, 1992); and two different substitutions at codon 243 (R243W, R243Q; Onigata *et al.*, 1995; Pohlenz *et al.*, 1996). Here, we report five novel TR β mutations (A268D, Δ 276I, T277A, A279V, R282S), together with the identification of known mutations (A243T, R243W, R243Q), in further families with RTH which constitute a third cluster of abnormalities localizing to a distinct part ($\alpha\alpha$ 234–282) of the (E) domain of the receptor. We have characterized these mutants extensively to determine their functional properties. When tested with reporter genes containing three different configurations of TRE, the extent to which their transcriptional function was impaired (Figure 1) was correlated inversely with ligand-binding affinities with the notable exception of T277A.

Identification of a role for helix 3 in ligand-dependent transactivation and the recruitment of coactivators

One mutant, T277A, within the third cluster showed substantially reduced transactivation compared with the

wild-type maximum, despite levels of T3 sufficient to overcome its reduced ligand-binding affinity. Recently, mutants involving another codon in this region (R243W/Q) were found to exhibit a similar transcriptional phenotype (Yagi *et al.*, 1997) which was explicable on the basis of markedly lower ligand-binding affinities of DNA-bound versus free mutant proteins. However, the ready dissociation of T277A mutant homodimers (Figure 6B) and conformational change of T277A heterodimers (Figure 6A) in the presence of ligand makes a similar explanation unlikely, implying that other properties integral to receptor function might be impaired. We considered the possibility that T3-dependent dissociation of corepressor from this mutant might be disproportionately impaired, as described with mutants involving proline 453, which precedes the C-terminal amphipathic α -helix (Yoh *et al.*, 1997), and an unusual mutant, R383H, located outside the three clusters (Clifton-Bligh *et al.*, 1998). However, impaired corepressor release by T277A was consistent with its reduced K_a (Figure 2). In contrast, hormone-dependent recruitment of coactivators (SRC-1, ACTR) by T277A–RXR heterodimers on a direct repeat TRE was significantly attenuated (Figure 3A and B), which correlated with marked transcriptional impairment on this element. Furthermore, overexpressing SRC-1 restored the transcriptional function of this mutant (Figure 4), supporting our contention that defective coactivator recruitment limits its activity *in vivo*.

A number of studies have shown that the C-terminal amphipathic α -helix is important for ligand-dependent transactivation (AF-2) and coactivator recruitment both in TR and other nuclear receptors (Danielian *et al.*, 1992; Baretino *et al.*, 1994; Durand *et al.*, 1994; Tone *et al.*, 1994). We have reported a natural RTH mutant involving a leucine to valine substitution at codon 454 (L454V) in helix 12 of TR β LBD which exhibits similarly attenuated transactivation due to reduced ability to recruit putative coactivators, such as SRC-1 and RIP140, and that artificial mutations of this hydrophobic (L454A) and a neighbouring charged residue (E457A) abrogate both of these functions (Tone *et al.*, 1994; Collingwood *et al.*, 1997). The present study reveals that an additional residue, a threonine located at codon 277 in helix 3 (T277), is also critical for AF-2 activity and coactivator recruitment. Furthermore, an artificial TR β mutant (K288I) situated at the distal end of helix 3 has previously been observed to impair transactivation while preserving ligand binding (O'Donnell and Koenig, 1990). More recently, mutation of the homologous residue (K366A) in the oestrogen receptor has been shown to reduce SRC-1 binding dramatically (Henttu *et al.*, 1997).

In the crystal structure of hTR β those residues in helix 12 (L454, E457) and helix 3 (T277, K288) that are critical for transactivation are in close physical proximity (Figure 8). Furthermore, the side chains of each of these residues are solvent exposed and, as such, are potentially capable of mediating interactions with cofactors. We suggest that these residues might collectively constitute a coactivator-interaction surface. A parallel crystallographic study supports this hypothesis, showing that a peptide containing an LXXLL motif, characteristic of the receptor-interacting domains of many coactivators (Heery *et al.*, 1997), binds the TR in a hydrophobic cleft bordered

Fig. 8. Location of key residues within the TR β ligand-binding domain that have been shown to be critical for transactivation function and/or coactivator recruitment in TR and other nuclear receptors. The ligand (T3) is shown in magenta. Side chains of residues in helix 3 (T277, K288), helix 5 (K306) and helix 12 (L454, E457) which may contribute to a receptor–coactivator interface are depicted.

by residues from helices 3, 5 and 12 (R.Wagner and B.Darimont, personal communication).

Preserved DNA-binding, heterodimerization, silencing and corepressor interaction are characteristic of mutations in the third cluster

The observation that individuals heterozygous for a deletion of a single TR β allele did not exhibit RTH (Takeda *et al.*, 1992) led to the hypothesis that mutant receptors in this syndrome were not simply functionally impaired, but were also capable of inhibiting wild-type receptor function in a dominant negative manner. A number of studies have shown that dominant negative activity is an intrinsic property of RTH mutants within the two previously recognized clusters (Sakurai *et al.*, 1990; Chatterjee *et al.*, 1991; Meier *et al.*, 1993; Zavacki *et al.*, 1993; Collingwood *et al.*, 1994; Hayashi *et al.*, 1994; Kitajima *et al.*, 1995). We have tested mutants located in the third cluster and find that they also exert dominant negative inhibitory effects when cotransfected with wild-type TR on three different TREs (Figure 5A–C). Previous studies have shown that three properties are essential for dominant negative activity of mutant receptors, DNA binding, dimerization with RXR and corepressor recruitment, such that the introduction of artificial mutations which abolish these functions abrogates dominant negative inhibition (Nagaya *et al.*, 1992; Nagaya and Jameson, 1993; Collingwood *et al.*, 1994; Yoh *et al.*, 1997). Although the hinge and D domain of TR have been implicated previously in DNA binding, heterodimerization and corepressor interaction, we found that without exception all mutants within the third cluster retained the ability to bind to DNA and heterodimerize with RXR on palindromic, direct or everted repeat TREs (Figure 6A–C). Moreover, they silence basal transcription *in vivo* (Figure 7A) and recruit corepressor

Fig. 9. (A) The third cluster of TR mutations and surrounding domains. The amino acid sequence of hTR β from residues 103 to 311 encompassing the DNA-binding, hinge and D domains of the receptor are shown. The shaded areas denote an α -helical C-terminal extension of the DBD delineated in the TR–RXR crystal structure (Rastinejad *et al.*, 1995), the CoR box implicated in recruitment of corepressor (Hörlein *et al.*, 1995) and a signature motif conserved amongst nuclear receptors and involved in stabilizing the canonical fold of the LBD (Wurtz *et al.*, 1996). The third cluster of RTH mutations extends from $\alpha\alpha$ 234–282. Non-RTH mutants which interfere with either corepressor (P214R, AHTm) or coactivator interaction (K288I, K306A) are italicized and the beginning of the next RTH mutation cluster at M310 is boxed. (B) Mapping of the third cluster of RTH mutations onto the TR β crystal structure. Ribbon drawing of the ligand binding domain with α -helices and coil conformations shown in grey. The hormone (magenta) is depicted as a space-filling model. The boundaries of the three clusters within which RTH mutations localize are coloured: brown (residues 310–353), blue (residues 429–461) and green (residues 234–282). The side chains of mutated codons within the third cluster are shown, forming another boundary to the ligand binding cavity. Receptor regions that are devoid of naturally occurring mutations are marked: the signature motif (boxed) involved in folding of the LBD and part of helix 11 (arrowed) mediating dimerization. The CoR box involved in corepressor binding is bracketed and the direction of the α -helical extension of the DBD is arrowed.

comparably with wild-type TR *in vitro* (Figure 7B). Analysis of the boundaries of the third cluster suggest that there are important functional constraints which explain the lack of naturally occurring mutations beyond it (Figure 9A). The crystal structure of the DNA-bound TR–RXR heterodimer has shown that the region proximal to the third cluster forms an α -helix interacting with the minor groove (Rastinejad *et al.*, 1995) and furthermore, domain-swap experiments have suggested that this region enables monomeric DNA binding by TR (Zechel *et al.*, 1994). This C-terminal extension to the DNA-binding domain is followed by a region (residues 211–238; the CoR box) implicated in corepressor recruitment (Hörlein *et al.*, 1995). Indeed, a point mutant within this sequence (P214R) is impaired for silencing (Figure 7) and binding to SMRT (Chen and Evans, 1995) and abolishes the dominant negative activity of *v-erbA*, the oncogenic counterpart of TR (Damm and Evans, 1993). In addition, a triple point mutation (A228M/H229M/T232M) in this region disrupts binding to NCoR (Hörlein *et al.*, 1995) and reverses dominant negative inhibition by RTH mutants (Yoh *et al.*, 1997). One RTH mutant (A234T) lies within the CoR box but recruits corepressor normally, suggesting that not all residues in this region are critical for corepressor binding. C-terminal to the third cluster is a sequence ($\alpha\alpha$ 286–305) which is so highly conserved among nuclear receptors that it has been termed the ‘signature motif’. Recent studies have shown that this

region is critical for dimerization with RXR, not only for TR, but also for RAR and VDR (O’Donnell *et al.*, 1991; Rosen *et al.*, 1993; Whitfield *et al.*, 1995). Elucidation of the crystal structures of several nuclear receptor LBDs (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995) indicate that residues in this motif mediate interhelical interactions which stabilize the hydrophobic core. We speculate that mutations occurring in this region would not cause RTH because they would disrupt folding of the LBD- and RXR-binding and therefore not function as dominant negative inhibitors of wild-type TR.

A third boundary to the ligand-binding cavity in the crystal structure of TR β is provided by residues in the new cluster

Two clusters of mutations occurring in RTH ($\alpha\alpha$ 310–353; $\alpha\alpha$ 429–461) have been shown previously to border the ligand-binding cavity when mapped to their homologous residues in the crystal structure of TR α (Wagner *et al.*, 1995). When the mutations occurring in our third cluster are mapped onto the TR β crystal structure (Figure 9B) these residues are seen to form a further boundary to the ligand-binding pocket. Indeed, some mutations (Δ 276I, A279V) involve residues whose side chains contact ligand directly, an observation concordant with these mutations being particularly deleterious for hormone binding. Collectively, our studies of a third cluster of naturally occurring mutations in RTH have delineated new struc-

ture–function relationships for TR β and emphasize the value of studying RTH as a model for understanding transcriptional regulation by this nuclear receptor.

Materials and methods

Clinical and genetic analyses

Serum-free T4 (FT4) and free T3 (FT3) levels (shown in Table I) were measured with fluoroimmunoassay (FIA) assays using Delfia technology (Wallac, Milton Keynes, UK). TSH levels were determined with a sensitive 'second generation' assay method (Delfia, Wallac, Milton Keynes, UK). The intra- and interassay coefficients of variation were <10% throughout.

Blood samples were obtained from members of each kindred and leucocyte DNA was extracted using standard techniques. For each index case, exons 4–10 of the *hTR β* gene were amplified by PCR from genomic DNA with flanking intronic oligonucleotide primers and sequenced directly as described previously (Collingwood *et al.*, 1997). Each mutation was verified with at least two independent PCRs and sequencing reactions and family members were also genotyped by direct sequencing.

Expression vectors and reporter constructs

Receptor mutations were generated by site-directed mutagenesis of human *TR β* cDNA and verified by sequencing as described previously (Collingwood *et al.*, 1997). A eukaryotic vector containing the Rous sarcoma virus (RSV) enhancer and promoter was used to express wild-type and mutant human *TR β* cDNAs (Collingwood *et al.*, 1997). The reporter plasmids MAL-tk, PALtk and F2-tk contain direct, palindromic or everted repeat configurations of TRE upstream of the viral thymidine kinase (tk) promoter and luciferase (*LUC*) gene as described (Collingwood *et al.*, 1994).

The Gal4–TR expression vectors consist of the hormone-binding domains of wild-type or mutant TR β (residues 174–461) coupled to the DNA-binding domain (residues 1–147) of Gal4. The reporter UAS-TKLUC contains two 17mer Gal4 binding sites upstream of the TK promoter and luciferase (Collingwood *et al.*, 1994). The internal control plasmid BOS- β gal contains the elongation factor-1 α promoter driving expression of the *lacZ* gene. The expression vector pSG5-SRC-1 contains a human SRC-1 cDNA isolated from a B-cell library (Collingwood *et al.*, 1997).

T3-binding assays

Mutant receptor cDNAs were subcloned into pGEM 7z and used as templates for coupled *in vitro* transcription and translation with rabbit reticulocyte lysate (TNT, Promega, Southampton, UK). The T3-binding affinity of each mutant receptor was determined using a filter binding assay. *In vitro* translated receptor (2–4 μ l) was incubated for 1 h at 30°C in 500 μ l binding buffer (20 mM Tris–HCl, 50 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, pH 8.0) containing 0.01 nM [¹²⁵I]-T3 (specific activity 3000 μ Ci/ μ g) and unlabelled competitor ligand. Bound ligand was separated from free by filtering through Millipore HAWP02500 filters. Scatchard analysis was performed to generate affinity constants (K_a) and the results shown are the mean of three or more separate determinations. Where ligand binding was not detected, the mutants are denoted as 'BND'.

Cell culture and transfection assays

JEG-3 cells were grown in Optimum supplemented with 2% (v/v) fetal calf serum (FCS) and 1% (v/v) PSF (Gibco-BRL, Paisley, UK). Eighteen hours prior to transfection the medium was replaced with Optimum containing 2% resin-stripped FCS. Cells were transfected by a 4 h exposure to calcium phosphate with 2 μ g (MAL-tk, PAL-tk) or 4 μ g (F2-tk) of reporter plasmid, together with 100 ng of wild-type or mutant receptor expression vector and 200 ng of BOS- β gal. Following a 36 h incubation, cells were lysed in buffer containing 25 mM glycine–glycine, 15 mM MgSO₄, 4 mM NaEGTA, 1 mM DTT and 1% (v/v) Triton X-100, pH 7.8. Luciferase activity was measured using an Autolumat LB 953 luminometer (Berthold, Stevenage, UK). β -galactosidase activity was also determined and used to normalize luciferase values for transfection efficiency.

EBNA cells (293) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% (v/v) PSF (Gibco-BRL). Eighteen hours prior to transfection the medium was replaced with DMEM and 10% FCS depleted of thyroid hormones. Cells were transfected with 500 ng of UASTKLUC, 50 ng of wild-type or

mutant Gal4–TR fusion expression vector and 100 ng of BOS- β gal by a 4-h exposure to calcium phosphate and assayed for luciferase and β -galactosidase activity as described above.

Gel mobility shift assays

The nucleotide sequences of the ³²P-labelled oligonucleotide duplexes used in gel mobility shift assays and the methodology were essentially as described previously (Collingwood *et al.*, 1994). ³⁵S-labelled mutant proteins were synthesized using a non-coupled transcription and translation system, according to the manufacturer's recommended protocol (Promega, Southampton, UK). Equal volumes of translation product were subjected to SDS–PAGE and the major bands quantified using an Instant Imager (Packard). Equal counts of each mutant receptor (2–4 μ l) were pre-incubated on ice for 20 min in buffer (20 mM HEPES, 50 mM KCl, 10% glycerol, 2 mM dithiothreitol, pH 7.8) in the presence of 1 μ g poly(dI–dC). *In vitro* translated hRXR α and triiodo-L-thyronine (T3) were also present where specified. The total amount of reticulocyte lysate in each assay was standardized by the addition of non-programmed translation mixture. ³²P-labelled oligonucleotides were added followed by a further 15-min incubation in a total volume of 20 μ l. In addition, where specified, bacterially expressed and purified GST fusions containing the receptor-interactive domains of SRC-1 [GST–SRC'; residues 570–780; 2.4 μ g/lane (Heery *et al.*, 1997)] or ACTR [GST–ACTR'; residues 621–821; 0.6 μ g/lane (Chen *et al.*, 1997)] were added. The protein–DNA complexes were then analysed using polyacrylamide gel electrophoresis (5% T, 3.3% C) in 0.5 $\times$ TBE (44 mM Tris, 44 mM boric acid, 1 mM EDTA). The assays were replicated using mutant proteins from at least two independent translation reactions.

Protein interaction assays

For the interaction of mutant receptors with the corepressor, a fusion protein (GST–C-SMRT) containing essentially the C-terminus of SMRT (Chen and Evans, 1995) was expressed bacterially, purified and incubated with radiolabelled wild-type or mutant receptors synthesized *in vitro* as described above. Aliquots of the immobilized fusion protein were incubated with the receptors in HEMG buffer (Yoh *et al.*, 1997) in the presence or absence of 1 μ M T3 for 2 h at 4°C then washed three times with NETN buffer (Collingwood *et al.*, 1997) and analysed by SDS–PAGE. Gels were Coomassie stained to check for equal loading of fusion protein, then exposed to autoradiography.

Acknowledgements

We thank R.Evans and M.Parker for the GST–ACTR' and GST–SRC' vectors respectively. This work was supported by the Wellcome Trust and MRC (UK). C.M. is a Wellcome Advanced Training Fellow. R.C.-B. is a Commonwealth Foundation Research Scholar. M.G. is a Wellcome Training Fellow.

References

- Adams, M., Matthews, C., Collingwood, T.N., Tone, Y., Beck-Peccoz, P. and Chatterjee, V.K.K. (1994) Genetic analysis of 29 kindreds with generalized and pituitary resistance to thyroid hormone. *J. Clin. Invest.*, **94**, 506–515.
- Barettino, D., Vicanco Ruiz, M.M. and Stunnenberg, H.G. (1994) Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J.*, **13**, 3039–3049.
- Beck-Peccoz, P., Chatterjee, V.K.K., Chin, W.W., DeGroot, L.J., Jameson, J.L., Nakamura, H., Refetoff, S., Usala, S.J. and Weintraub, B.D. (1994) Nomenclature of thyroid hormone receptor β gene mutations in resistance to thyroid hormone: consensus statement from the First Workshop on Thyroid Hormone Resistance, July 10–11, 1993, Cambridge, UK. *J. Clin. Endocrinol. Metab.*, **78**, 990–993.
- Behr, M. and Loos, U. (1992) A point mutation (Ala 229 to Thr) in the hinge domain of the *c-erbA β* thyroid hormone receptor gene in a family with generalized thyroid hormone resistance. *Mol. Endocrinol.*, **6**, 1119–1126.
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR. *Nature*, **375**, 377–382.
- Cavaillès, V., Dauvois, S., L'Hors, F., Lopez, G., Hoare, S., Kushner, P.J. and Parker, M.G. (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.*, **14**, 3741–3751.

- Chakravarti,D., LaMorte,V.J., Nelson,M.C., Nakajima,T., Schulman,I.G., Juguilon,H., Montminy,M. and Evans,R.M. (1996) Role of CBP/P300 in nuclear receptor signalling. *Nature*, **383**, 99–103.
- Chatterjee,V.K.K., Nagaya,T., Madison,L.D., Datta,S., Rentoumis,A. and Jameson,J.L. (1991) Thyroid hormone resistance syndrome: inhibition of normal receptor function by mutant thyroid hormone receptors. *J. Clin. Invest.*, **87**, 1977–1984.
- Chen,H., Lin,R.J., Schiltz,R.L., Chakravarti,D., Nash,A., Nagy,L., Privalsky,M.L., Nakatani,Y. and Evans,R.M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell*, **90**, 569–580.
- Chen,J.D. and Evans,R.M. (1995) A transcriptional corepressor that interacts with nuclear hormone receptors. *Nature*, **377**, 454–457.
- Clifton-Bligh,R.J., de Zegher,F., Wagner,R.L., Collingwood,T.N., Francois,I., van Helvoirt,M. and Chatterjee,V.K.K. (1998) A novel TR β mutation (R383H) in resistance to thyroid hormone predominantly impairs corepressor release and negative transcriptional regulation. *Mol. Endocrinol.*, **12**, 609–621.
- Collingwood,T.N., Adams,M., Tone,Y. and Chatterjee,V.K.K. (1994) Spectrum of transcriptional, dimerization and dominant negative properties of twenty different mutant thyroid hormone β -receptors in thyroid hormone resistance syndrome. *Mol. Endocrinol.*, **8**, 1262–1277.
- Collingwood,T.N. *et al.* (1997) A natural transactivation mutation in the thyroid hormone β receptor: impaired interaction with putative transcriptional mediators. *Proc. Natl Acad. Sci. USA*, **94**, 248–253.
- Damm,K. and Evans,R.M. (1993) Identification of a domain required for oncogenic activity and transcriptional suppression by *v-erbA* and thyroid-hormone receptor. *Proc. Natl Acad. Sci. USA*, **90**, 10668–10672.
- Danielian,P.S., White,R., Lees,J.A. and Parker,M.G. (1992) Identification of a conserved region required for hormone-dependent transcriptional activation by steroid hormone receptors. *EMBO J.*, **11**, 1025–1033.
- Durand,B., Saunders,M., Gaudon,C., Roy,B., Losson,R. and Chambon,P. (1994) Activation function 2 (AF-2) of retinoic acid receptor and 9-*cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO J.*, **13**, 5370–5382.
- Forman,B.M. and Samuels,H.H. (1990) Interactions among a family of nuclear receptors: the regulatory zipper model. *Mol. Endocrinol.*, **4**, 1293–1301.
- Hayashi,Y., Sunthornthepvarakul,T. and Refetoff,S. (1994) Mutations of CpG dinucleotides located in the triiodothyronine (T3)-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. *J. Clin. Invest.*, **94**, 607–615.
- Heery,D.M., Kalkhoven,E., Hoare,S. and Parker,M.G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature*, **387**, 733–736.
- Heinzel,T. *et al.* (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature*, **387**, 43–48.
- Henttu,P.M.A., Kalkhoven,E. and Parker,M.G. (1997) AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors. *Mol. Cell. Biol.*, **17**, 1832–1839.
- Hörlein,A.J. *et al.* (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*, **377**, 397–404.
- Kamei,Y. *et al.* (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, **85**, 403–414.
- Kitajima,K., Nagaya,T. and Jameson,J.L. (1995) Dominant negative and DNA-binding properties of mutant thyroid hormone receptors that are defective in homodimerization but not heterodimerization. *Thyroid*, **5**, 343–353.
- Lazar,M.A. (1993) Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocr. Rev.*, **14**, 184–193.
- Mangelsdorf,D.J. and Evans,R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841–850.
- Meier,C.A. *et al.* (1992) Variable transcriptional activity and ligand binding of mutant β 1, 3,5,3'-triiodothyronine receptors from four families with generalised resistance to thyroid hormone. *Mol. Endocrinology*, **6**, 248–258.
- Meier,C.A., Parkison,C., Chen,A., Ashizawa,K., Meier-Heusler,S.C., Muchmore,P., Cheng,S.-Y. and Weintraub,B.D. (1993) Interaction of human β 1 thyroid hormone receptor and its mutants with DNA and retinoid X receptor. T3 response element-dependent dominant negative potency. *J. Clin. Invest.*, **92**, 1986–1993.
- Nagaya,T. and Jameson,J.L. (1993) Thyroid hormone receptor dimerization is required for dominant negative inhibition by mutations that cause thyroid hormone resistance. *J. Biol. Chem.*, **268**, 15766–15771.
- Nagaya,T., Madison,L.D. and Jameson,J.L. (1992) Thyroid hormone receptor mutants that cause resistance to thyroid hormone: evidence for receptor competition for DNA sequences in target genes. *J. Biol. Chem.*, **267**, 13014–13019.
- Nagy,L., Kao,H.-Y., Chakravarti,D., Lin,R.J., Hassig,C.A., Ayer,D.E., Schreiber,S.L. and Evans,R.M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A and histone deacetylase. *Cell*, **89**, 373–380.
- O'Donnell,A.L. and Koenig,R.J. (1990) Mutational analysis identifies a new functional domain of the thyroid hormone receptor. *Mol. Endocrinol.*, **4**, 715–720.
- O'Donnell,A.L., Rosen,E.D., Darling,D.S. and Koenig,R.J. (1991) Thyroid hormone receptor mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein. *Mol. Endocrinol.*, **5**, 94–99.
- Oñate,S.A., Tsai,S.Y., Tsai,M.-J. and O'Malley,B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science*, **270**, 1354–1357.
- Onigata,K., Yagi,H., Sakurai,A., Nagashima,T., Nomura,Y., Nagashima,K., Hashizume,K. and Morikawa,A. (1995) A novel point mutation (R243Q) in exon 7 of the *c-erbA* β thyroid hormone receptor gene in a family with resistance to thyroid hormone. *Thyroid*, **5**, 355–358.
- Parrilla,R., Mixson,A.J., McPherson,J.A., McClaskey,J.H. and Weintraub,B.D. (1991) Characterization of seven novel mutations of the *c-erbA* β gene in unrelated kindreds with generalized thyroid hormone resistance: evidence for two 'hot spot' regions of the ligand binding domain. *J. Clin. Invest.*, **88**, 2123–2130.
- Pohlenz,J., Schonberger,W., Wemme,H., Winterpacht,A., Wirth,S. and Zabel,B. (1996) New point mutation (R243W) in the hormone binding domain of the *c-erbA* β 1 gene in a family with generalized resistance to thyroid hormone. *Hum. Mutat.*, **7**, 79–81.
- Rastinejad,F., Perlmann,T., Evans,R.M. and Sigler,P. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature*, **375**, 203–211.
- Refetoff,S., Weiss,R.E. and Usala,S.J. (1993) The syndromes of resistance to thyroid hormone. *Endocr. Rev.*, **14**, 348–399.
- Renaud,J.-P., Rochel,N., Ruff,M., Vivat,V., Chambon,P., Gronemeyer,H. and Moras,D. (1995) Crystal structure of the RAR-ligand-binding domain bound to all-*trans* retinoic acid. *Nature*, **378**, 681–689.
- Rosen,E.D., Beninghof,E.G. and Koenig,R.J. (1993) Dimerization interfaces of thyroid hormone, retinoic acid, vitamin D and retinoid X receptors. *J. Biol. Chem.*, **268**, 11534–11541.
- Sakurai,A., Miyamoto,T., Refetoff,S. and DeGroot,L.J. (1990) Dominant negative transcriptional regulation by a mutant thyroid hormone receptor β in a family with generalised resistance to thyroid hormone. *Mol. Endocrinol.*, **4**, 1988–1994.
- Takeda,K., Sakurai,A., De Groot,L.J. and Refetoff,S. (1992) Recessive inheritance of thyroid hormone resistance caused by complete deletion of the protein-coding region of the thyroid hormone receptor- β gene. *J. Clin. Endocrinol. Metab.*, **74**, 49–55.
- Tone,Y., Collingwood,T.N., Adams,M. and Chatterjee,V.K.K. (1994) Functional analysis of a transactivation domain in the thyroid hormone β receptor. *J. Biol. Chem.*, **269**, 31157–31161.
- Voegel,J.J., Heine,M.J.S., Zechel,C., Chambon,P. and Gronemeyer,H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.*, **15**, 3667–3675.
- Wagner,R.L., Apriletti,J.W., McGrath,M.E., West,B.L., Baxter,J.D. and Fletterick,R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature*, **378**, 690–697.
- Whitfield,G.K., Hsieh,J.-C., Nakajima,S., MacDonald,P.N., Thompson,P.D., Jurutka,P.W., Haussler,C.A. and Haussler,M.R. (1995) A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. *Mol. Endocrinol.*, **9**, 1166–1179.
- Wurtz,J.-M., Bourguet,W., Renaud,J.-P., Vivat,V., Chambon,P., Moras,D. and Gronemeyer,H. (1996) A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struct. Biol.*, **3**, 87–94.

- Yagi,H., Pohlenz,J., Hayashi,Y., Sakurai,A. and Refetoff,S. (1997) Resistance to thyroid hormone caused by two mutant thyroid hormone receptors β , R243Q and R243W, with marked impairment of function that cannot be explained by altered *in vitro*, 3,5,3'-triiodothyronine binding affinity. *J. Clin. Endocrinol. Metab.*, **82**, 1608–1614.
- Yen,P.M. and Chin,W.W. (1994) Molecular mechanisms of dominant negative activity by nuclear hormone receptors. *Mol. Endocrinol.*, **8**, 1450–1454.
- Yoh,S.M., Chatterjee,V.K.K. and Privalsky,M.L. (1997) Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. *Mol. Endocrinol.*, **11**, 470–480.
- Zavacki,A.M., Harney,J.W., Brent,G.A. and Larsen,P.R. (1993) Dominant negative inhibition by mutant thyroid hormone receptors is thyroid response element and receptor isoform specific. *Mol. Endocrinol.*, **7**, 1319–1330.
- Zechel,C., Shen,X.-Q., Chambon,P. and Gronemeyer,H. (1994) Dimerization interfaces formed between the DNA binding domains determine the cooperative binding of RXR/RAR and RXR/TR heterodimers to DR5 and DR4 elements. *EMBO J.*, **13**, 1414–1424.
- Zhang,J., Zamir,I. and Lazar,M.A. (1997) Differential recognition of liganded and unliganded thyroid hormone receptor by retinoid X receptor regulates transcriptional repression. *Mol. Cell. Biol.*, **17**, 6887–6897.

Received May 18, 1998; revised and accepted June 25, 1998