Identification and characterization of a novel *de novo* mutation (L346V) in the thyroid hormone receptor β gene in a family with generalized thyroid hormone resistance

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Abstract

We have investigated an Italian family with generalized resistance to thyroid hormone (RTH), consisting of two individuals with elevated serum thyroid hormones (TH) and a non-suppressed TSH, together with unaffected family members, for a mutation in the thyroid hormone receptor β gene (hTRβ). We have identified a single nucleotide substitution (1321 CTT to GTT) corresponding to a leucine to valine substitution at codon 346 (L346V) in the predicted protein. The index case and her affected child are heterozygous for the receptor defect, with normal sequence in unaffected family members. Furthermore, both parents of the index case were unaffected, suggesting that the mutation had arisen *de novo*. When expressed *in vitro*, the L346V mutant receptor showed a marked reduction in its affinity for tri-iodothyronine (T3), impaired ligand-dependent transactivation and potent dominant negative activity. Its functional impairment could not be alleviated, even at supraphysiological concentrations of T3, suggesting that the mutation might interfere with the intrinsic ligand-dependent transactivation function (AF-2) located in the hormone binding domain of hTRβ.

Finally, the presence of the L346V mutation in the son of the propositus, who died from complications associated with congenital heart disease, raises the possibility that RTH might have contributed to the pathogenesis or severity of the latter.

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Introduction

Resistance to thyroid hormone (RTH) is a rare syndrome characterized by high levels of circulating thyroid hormones (TH), a non-suppressed thyrotrophin (TSH) and variable tissue hyposensitivity to TH (1, 2). This syndrome is associated with diverse mutations in the thyroid hormone receptor β (hTRβ) gene which encodes a ligand-dependent nuclear transcription factor that modulates the transcription of target genes by interacting with thyroid response elements (TREs) in their promoter region (3–6). The TRβ gene undergoes alternate splicing to generate two receptor isoforms, TRβ1 and TRβ2, the latter differing from β1 in the N-terminus and being mainly expressed in the pituitary (7). TRβ1 consists of 10 exons (8); the mutations detected hitherto are located in the last 4 exons coding for the hinge region and the ligand-binding domain of the receptor. The majority of these mutations result in reduced tri-iodothyronine (T3)-binding and/or impairment of transactivation. In addition, the mutant receptor is able to inhibit wild-type receptor action when they are coexpressed *in vitro*. This dominant negative inhibitory effect is consistent with the autosomal dominant inheritance of RTH (1). Mutations in the hTRβ gene have been identified in more than 100 families, but only in 18% of the families studied does the syndrome appear to be sporadic. In fact, the majority of these sporadic cases appear to represent *de novo* mutations. To date, 56 different mutations have been described and listed in the on-line RTH registry of the University of Chicago (9). The degree of resistance to TH varies in different organs, resulting in different clinical features ranging from hyper- to hypothyroidism, consistent with retention of tissue sensitivity or severe resistance respectively (1, 10–13).

Here, we report a novel missense mutation in exon 9 of hTRβ1, resulting in a leucine to valine substitution (L346V) in an Italian patient with generalized RTH. This mutant receptor showed a marked decrease in its affinity for T3, impaired function, and potent dominant negative activity. Family studies demonstrated that this was a *de novo* mutation, as both parents were unaffected. The mutation was also transmitted to the offspring of the index case, who exhibited features of RTH and died at the age of 12 from congenital heart disease.
disease, raising the possibility that RTH might have contributed to his death.

Subjects and methods

Subjects

In January 1980, S.L., a 25-year-old Italian woman was referred to our outpatient clinic with a goitre and symptoms of anxiety, 4 months after completing a long-term course of antithyroid drug therapy. The goitre was first noted at the age of 10, initially with no other symptoms of thyroid dysfunction. She remained in good health until the end of 1977 when anxiety and fatigue began. In January 1978 the patient was evaluated elsewhere and treated for 18 months with methimazole leading to an improvement in the above symptoms. On examination she appeared well but was hyperactive. Her height was 152 cm and her weight was 52 kg. Her resting pulse was 74 beats/min with a blood pressure of 120/80 mmHg. A grade 2 ejection systolic murmur was present. The skin was moist and warm. The thyroid gland was enlarged (3 times the normal size) but not nodular in consistency. There were no signs of ophthalmopathy. Thyroid function tests were as follows: total T4 (TT4) 186 nmol/l, total T3 (TT3) 5.18 mmol/l, T3 resin uptake (T3RU) 62.3%, free T4 index (FT4I) 18.2, free T3 index (FT3I) 432; thyroid antibodies (Abs) were absent (see Table 1 for normal ranges). The 24-h radioiodine uptake (RAIU) was 65% and scan showed an enlarged gland with homogeneous uptake. A chest X-ray showed bilateral compression of the tracheal wall. A diagnosis of toxic diffuse goitre was made and a second 18-month course of methimazole was prescribed. Unfortunately, the patient was then lost to follow-up. Because of poor compliance with this therapy and an increase in the goitre with symptoms of tracheal compression, the patient was submitted, elsewhere, to subtotal thyroidectomy in December 1981. In January 1983, at the 42nd week of pregnancy, the patient delivered a male child in normal circumstances.

The patient was referred back to our outpatient clinic in May 1993 for reassessment. In the previous four months she had not been taking drugs known to interfere with thyroid function. She was 39 years old and weighed 58 kg. Thyroid function tests were as follows (see Table 1): FT4I 15.7, FT3I 253, FT4 26.6 pmol/l, FT3 10.2 pmol/l, TSH 19.6 mU/l; thyroid Abs, including TSH receptor Abs, were negative.

In view of the elevated serum TH with inappropriate TSH secretion, she was admitted for further investigations. Thyroid function was evaluated by measurement of T3RU (Trilude kit, Miles Italiana, Ames Division, Milan, Italy), TT4 (AIA-Pack T4, Eurogenetics, Turin, Italy) and FT4I (calculated as the product of TT4 and T3RU), TT3 (AIA-Pack TT3, Eurogenetics) and FT3I (calculated as the product of TT3 and TT3RU), FT4 (FT4 kit Liso-phase, Technogenetics s.r.l., Milan, Italy), FT3 (FT3 kit Liso-phase, Technogenetics s.r.l.), ultrasensitive TSH (AutoDelphia hTSH Ultra kit, Wallac, Pharmacia).

Table 1 Clinical and biochemical parameters of the index case with RTH.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basala</th>
<th>Basal</th>
<th>Post-T3</th>
<th>Normal range</th>
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<tbody>
<tr>
<td>Total T4 (nmol/l)</td>
<td>186</td>
<td>163</td>
<td>82</td>
<td>60–120</td>
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<tr>
<td>Total T3 (nmol/l)</td>
<td>5.18</td>
<td>3.15</td>
<td>4.42</td>
<td>1.4–2.5</td>
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<td>T3 resin uptake (%)</td>
<td>62.3</td>
<td>61.9</td>
<td>62.1</td>
<td>35–63</td>
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<tr>
<td>Free T4 index</td>
<td>18.2</td>
<td>15.7</td>
<td>8.0</td>
<td>4–13</td>
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<tr>
<td>Free T3 index</td>
<td>432</td>
<td>253</td>
<td>375</td>
<td>100–208</td>
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<tr>
<td>Free T4 (pmol/l)</td>
<td>—</td>
<td>26.6</td>
<td>12.2</td>
<td>10–25</td>
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<tr>
<td>Free T3 (pmol/l)</td>
<td>—</td>
<td>10.2</td>
<td>15.0</td>
<td>4.0–7.4</td>
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<tr>
<td>TSH (mU/l)</td>
<td>3.2</td>
<td>19.6</td>
<td>11.3</td>
<td>0.4–3.7</td>
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<td>Molar alpha SU/TSH ratio</td>
<td>—</td>
<td>0.35</td>
<td>—</td>
<td>0.3–5.7</td>
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<tr>
<td>Peak TSH after TRH (mU/l)</td>
<td>—</td>
<td>190</td>
<td>93.1</td>
<td>3–30</td>
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<td>TgAb</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
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<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
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<td>15.6</td>
<td>15.6</td>
<td>14–35</td>
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<td>Urinary iodine excretion</td>
<td>50.7</td>
<td>54.8</td>
<td>56.2</td>
<td>20–100</td>
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<td>Cholesterol (nmol/l)</td>
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<td>3.6</td>
<td>3.4</td>
<td>&lt;5.2</td>
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<td>CK (U/l)</td>
<td>68</td>
<td>72</td>
<td>70</td>
<td>28–140</td>
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<td>24-h RAIU (%)</td>
<td>65</td>
<td>51</td>
<td>21</td>
<td>25–45</td>
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<td>BMR (KCal/day)</td>
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<td>1390</td>
<td>—</td>
<td>1250–1350</td>
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<tr>
<td>Resting pulse rate (beats/min)</td>
<td>75</td>
<td>62</td>
<td>80</td>
<td>60–90</td>
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<tr>
<td>Blood pressure (mmHg)</td>
<td>120/80</td>
<td>100/70</td>
<td>110/80</td>
<td>—</td>
</tr>
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</table>

a Initial evaluation made in January 1980, with no treatment. b Data obtained in May 1993, after thyroidectomy performed in 1981. c Following administration of 80 μg/day T3 for 10 days.

TgAb, antithyroglobulin Abs; TPOAb, antithyroperoxidase Abs.
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Milan, Italy), thyroid Abs (Serodia-ATG and Serodia-AMC, Fujirebio Inc., Tokyo, Japan), thyrotrophin-releasing hormone (TRH) stimulation test (levels of TSH were measured 0, 30 and 60 min after i.v. injection of 200 μg TRH (Ferring Arzneimittel GmbH, Kiel, Germany)), thyroxine-binding globulin (TBG) (CIS Biointernational, Gif-sur-Yvette, France), TSH alpha subunit (SU) (Bioclone, Marrickville, Australia) and molar TSH/alpha SU ratio, RAIU, thyroid scan and ultrasound. Several parameters of TH action in the periphery, including resting pulse rate, blood pressure, ECG, basal metabolic rate (BMR), sex hormone-binding globulin (SHBG) (Orion Diagnostics, Espoo, Finland), cholesterol, and creatinine kinase were evaluated. Urinary iodine excretion was also measured, using an autoanalyzer apparatus (Technicon, Rome, Italy). After basal evaluation, a standard T₃ suppression test (20 μg q.d.s. for 10 days) was performed in order to determine the level of resistance in vivo.

Both parents, and other available family members, were also investigated from a clinical and biochemical point of view, in order to assess their thyroid status.

**Amplification and sequencing of the TRβ gene**

Genomic DNA was extracted from peripheral blood leucocytes of the propositus and other family members by the standard phenol/ethanol method. Exons 4 through 10 of the hTRβ1 gene were amplified by PCR, using specific primers complementary to intronic sequences flanking the various hTRβ exons. Reactions were carried out with Taq polymerase (Promega Corp., Madison, WI, USA), using 1 μg genomic DNA and 50 pmol of each primer in a 50 μl reaction volume, containing 10 mmol Tris–HCl pH 9, 50 mmol KCl, 2 mmol MgCl₂, 200 μmol dNTPs, and 2.5 U Taq polymerase. After an initial 5 min denaturation at 94 °C, the sample was cycled for 30 s at 94 °C, 60 s at 57 °C, and 90 s at 72 °C for 30 cycles. PCR products were checked on a 2% agarose gel and visualized with U.V. light after staining with ethidium bromide. If the sequences flanking the various hTR β exons were confirmed by at least three independent PCR and sequencing reactions.

**Allele-specific amplification of genomic DNA**

Fifty pmol of three primers (primer exon 9 sense: 5'-TGCTGACATGAACTGGTTCT-3', primer exon 9 anti-sense wild-type: 5'-GGCTCGACACCCACCCCAAG-3', primer exon 9 anti-sense mutant type: 5'-GGCTCTGA-

CACCACCCAAAC-3') were annealed to 1 μg genomic DNA in two separate reactions and amplified for 30 cycles by PCR, following the protocol described by Takeda et al. (14). The PCR products were separated by 2% agarose gel and visualized using ethidium bromide staining. Positive and negative control reactions were included, and each experiment was repeated three times.

**Mutagenesis and plasmids**

The L346V mutation was generated by site-directed mutagenesis of the wild-type hTRβ1 cDNA in M13 mp18 as described previously (15). Both wild-type and mutant receptor cDNAs were then subcloned into a vector downstream of the Rous sarcoma virus (RSV) enhancer and promoter for transient transfection assays. The reporter plasmid MAL-TKLUC contains a positively regulated thyroid response element (TRE) from the malic enzyme gene inserted upstream of the viral thymidine kinase gene promoter and luciferase cDNA. The internal control plasmid BOS-βgal contains the elongation factor-1α promoter driving expression of the β-galactosidase gene (16).

**Ligand binding assays**

Wild-type and mutant receptor proteins were synthesized from cDNA templates in pGEM7Z by coupled transcription and translation in vitro (TNT system, Promega, Southampton, UK). The ligand binding affinity of each receptor for [125I]T₃ was measured using a filter binding assay described previously (10). Three separate experiments were performed on independently generated samples of receptor proteins, and binding affinity constants (Kᵦ) were calculated from Scatchard plots of the bound and free fractions.

**Cell culture and transfection assays**

JEG-3 cells were grown in OptiMEM (GIBCO BRL, Paisley, Scotland) containing 2% (vol/vol) fetal calf serum and 1% (vol/vol) penicillin, streptomycin, fungizone (GIBCO BRL) and 18 h prior to transfection the medium was changed to OptiMEM with 2% charcoal-stripped fetal calf serum. Twenty-four-well plates of cells were transfected by a 5-h exposure to calcium phosphate. After a further 36 h, the cells were lysed and extracts assayed for luciferase and β-galactosidase activity.

**Paternity studies**

Paternity was investigated by fingerprint analysis, having obtained the subjects’ informed consent. DNA was extracted from peripheral blood lymphocytes and digested with Hinf I (GIBCO BRL). The material was electrophoresed on a 0.8% agarose gel, transferred onto a nitrocellulose membrane, and hybridized with a 32P
M13 probe, as described by Vassart et al. (17). The bands were analysed after autoradiography at \(-80^\circ\)C for 12–18 h.

Results

Clinical data from the index case

In May 1993, S L, aged 39, appeared in good health, a cervical scar was present and no thyroid tissue was felt on palpation. The resting pulse was 62 beats/min and the blood pressure 110/60 mmHg. No other physical abnormalities were found. All routine laboratory tests were normal. Results of thyroid function tests and selected parameters related to the peripheral effects of TH are reported in Table 1 and confirm elevated TH with inappropriate TSH secretion. However, the glyco-protein hormone alpha subunit/TSH molar ratio was normal. The 24-h RAIU was 51% and the thyroid scan showed a bilateral remnant which measured 5 ml on ultrasound. A resting ECG was normal. The BMR was 1390 kCal/day (107%). A pituitary CT scan was normal. A T\(_3\) suppression test (20 \(\mu\)g T\(_3\) every 6 h for 10 days) was performed (see Table 1) and showed a 59% decrease in the 24-h RAIU, 42.3% decrease in basal TSH but only a 51% reduction in the peak TSH response following TRH, compared with a mean suppression of 82% in four non-resistant hypothyroid patients and >75% in the group of non-resistant subjects described by Sarne et al. (18). We also had the opportunity to measure TSH in previous serum samples from the proposita stored at \(-20^\circ\)C. The results always showed a detectable serum TSH associated with increased TH levels. In particular, her serum TSH in a sample from January 1980, in the absence of any therapy, was 3.2 mU/l. On the basis of the overall laboratory and clinical evaluation a diagnosis of resistance to TH was made.

Family studies

Figure 1 shows a partial pedigree consisting of family members that have been investigated. The proposita and her son had a biochemical phenotype of RTH, whereas both parents and the other available family members had normal clinical and laboratory findings. The child of the proposita was born in January 1983 and was subsequently found to have a congenital cardiac defect in the form of Ebstein’s anomaly of the tricuspid valve. Although no overt symptoms of hyperthyroidism were documented in childhood, he died at the age of 12 from a severe supraventricular tachyarrhythmia and associated cardiac failure. His thyroid function test results were as follows; TSH 10.1 mU/l, FT\(_4\) 37.5 pmol/l, FT\(_3\) 10.6 pmol/l, TBG 29 mg/l, and an absence of thyroid Abs, thus showing the characteristic biochemical pattern of RTH. His father is in good health and does not show any thyroidal or cardiac abnormality.

In order to exclude paternal misidentification or unrevealed adoption, paternity studies were performed on the proposita and her parents, using a DNA fingerprinting technique. The hypervariable polymorphic pattern detected in the DNA of these three individuals established paternity with a probability higher than 99.95%.

TR\(_\beta\) gene sequencing

Figure 2 shows some nucleotide sequence of exon 9 of hTR\(_\beta\)1 from an unaffected member of the family (W T) and from the proposita (M T). At nucleotide 1321 of M T there was a C to G transition corresponding to a leucine to valine at codon 346 of the amino acid sequence of hTR\(_\beta\)1. Heterozygosity for the mutation was supported by the presence of wild-type sequence at the same site, which was confirmed by determining the sequence of both DNA strands from several independently amplified PCR products. The same mutation was found in genomic DNA from the offspring, but it was absent in the parents and in the paternal grandfather.

Allele-specific amplification

In order to confirm the genetic defect found in exon 9 of the proposita and her son, we screened all available family members using an allele-specific amplification

![Figure 1](https://example.com/figure1.png)

**Figure 1** Pedigree of the family with RTH. The circles and squares indicate female and male family members respectively. Open symbols represent untested members, solid symbols the affected members, shaded symbols the unaffected individuals, and barred symbols the deceased relatives.

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

**Figure 2** Sequence analysis of wild-type and mutant hTR\(_\beta\)1 exon 9. Part of the sequence of exon 9 from the index case (MT) and from an unaffected family member (WT) are shown. A single nucleotide substitution at position 1321 (arrow) changes codon 346 from leucine to valine in MT, who is heterozygous for this mutation. Only the wild-type sequence is present in the unaffected individual.
technique. The mutant, in fact, does not create or destroy any restriction site. Separate PCR reactions using allele-specific primers containing the normal and mutant nucleotides at the 3'-position were performed and the results are shown in Fig. 3. Ethidium bromide staining of the 2% agarose gel revealed a band specific for the mutant allele only in the index case and in her son. In all other family members only the primers containing wild-type sequence amplified exon 9 of hTRβ1.

**Ligand binding affinity**

The T3 binding affinity of the L346V mutant was evaluated by synthesizing the wild-type and mutant receptor proteins in vitro as described. The wild-type hTRβ1 protein exhibited an affinity constant (Kₐ) of 0.43×10⁻¹⁰ mol/l (S.E.M=0.011) and in comparison the Kₐ of the L346V mutant was significantly reduced at 0.01×10⁻¹⁰ mol/l (S.E.M=0.004).

**Functional assays**

The function of wild-type and mutant receptors was tested by assaying their ability to activate a reporter gene (MAL-TKLUC) containing a positive TRE in a hormone-dependent manner. In comparison to the wild-type receptor, the L346V mutant exhibited impaired function with a right-shifted activation profile. Furthermore, the mutant was unable to attain maximal wild-type levels of function even at a T3 concentration (1000 nmol/l) sufficient to overcome its reduced hormone binding affinity (Fig. 4).

Having established the functional properties of the mutant receptor when expressed alone, its ability to modulate the activity of wild-type receptor was then assessed. The wild-type and mutant receptors were coexpressed in an equal ratio with MAL-TKLUC and reporter gene activity assayed at either low (1 nmol/l) or high (1000 nmol/l) T3 concentrations. As expected, L346V displayed potent dominant negative activity at the lower hormone concentration, inhibiting wild-type receptor action from 50% to 10%. However, the presence of 1000 nmol/l T3 was still associated with a significant dominant negative effect (Fig. 5), in keeping with the inability of high concentrations of ligand to restore mutant receptor function fully.
We have identified a novel mutation in the thyroid hormone receptor β gene in a kindred with RTH. Affected individuals in this family are heterozygous for a leucine to valine substitution at codon 346 of the hTRβ gene. The mutation was identified in an Italian woman with generalized RTH, who had previously been diagnosed as having a toxic diffuse goitre for which she was initially treated with antithyroid drugs, and later by subtotal thyroidectomy. This history underscores the difficulties in diagnosing RTH, particularly when, as reported here, measurement of serum TSH was not a routine part of the patient’s evaluation. Thus, the paucity of specific clinical manifestations in RTH, together with features such as goitre and hyperthyroid symptoms and elevated thyroid hormones has often prompted an incorrect diagnosis of hyperthyroidism in the past (1). In our case, due to the failure to recognise inappropriately normal TSH levels, treatment aimed at normalizing TH levels was prescribed. The subsequent finding of a high serum TSH, in the face of persistently elevated serum TH, eventually led to the correct diagnosis. At the time of investigation in our Department in 1993, the index case had undergone a subtotal thyroidectomy 12 years previously. Interestingly, while a recurrence of goitre after surgery has been described frequently in RTH, our case showed only a small glandular remnant of approximately 5 ml, without obvious symptoms or signs of hypothyroidism. However, serum TSH levels of 19.6 mU/l were well within the hypothyroid range and circulating TH levels were lower than those prior to surgery. It is worth noting that, despite a six-fold increase in TSH (post-thyroidectomy versus basal), none of the peripheral markers of TH action were altered significantly, suggesting an apparent compensated state. Alternatively, this finding could indicate either an inability of peripheral markers to discriminate between euthyroid and mildly hypothyroid states, or a lower sensitivity of the pituitary thyrotrophs to the effects of TH compared with other target tissues. Spontaneous fluctuations in the degree of resistance to TH in a given individual during his lifetime are also reported (19).

Paternity studies performed on the proposita and her parents excluded paternal misidentification or unrevealed adoption and established that the mutation found in her TRβ gene represented a de novo mutation. Heterozygosity for the L346V mutation was not evident in unaffected relatives, but was transmitted to her affected son, showing concordance between genotype and biochemical phenotype. The fact that this child also had congenital heart disease and died at the age of 12 from a severe supraventricular tachyarrhythmia and cardiac failure is also noteworthy. In a recent review by Brucker-Davis et al. (11) of the clinical features of RTH, 6 cases of arrhythmia and 8 cases of valve defects were found in those with the syndrome, while there were none in individuals without resistance. This finding raises the possibility that mutations in the hTRβ gene might predispose to cardiac malformations. It is also possible that RTH exacerbated the effects of the congenital anomaly. It has been suggested that the myocardium is less resistant to TH than other tissues in this disorder, perhaps due to the fact that TRα1 is more highly expressed than TRβ1 in this organ (20, 21), such that it retains sensitivity to excess circulating TH.

Studies in vitro indicate that the T3 binding affinity of the mutant receptor is significantly reduced. The recent elucidation of the crystal structure of the ligand binding domain of TRα provides a structural rationale for this finding (22). Thus, Leu 292 in TRα which is homologous to Leu 346 in TRβ, is part of a loop between two α-helices (helix 7 and 8) and forms part of the boundary of the hormone binding cavity. Together with other residues (Phe 218, Leu 276, Leu 287), this amino acid constitutes a hydrophobic environment around the ether oxygen of the ligand. Interestingly, the homologous leucine to valine substitution at position 292 in TRα has been constructed and studied previously and ligand binding is also markedly impaired in this context (23).

In transfection assays the L346V mutant receptor exhibits impaired hormone-dependent reporter gene activation. However, its functional impairment cannot be compensated even in the presence of supraphysiological
(1000 nmol/l) concentrations of T3, sufficient to overcome its impaired ligand binding. In keeping with this observation, the mutant receptor also retains dominant negative activity at high T3 levels. These properties suggest that the L346V mutation might influence the intrinsic hormone-dependent transactivation function (AF-2) within the carboxyterminal domain of TRβ. With the recent identification of coactivators and corepressors which modulate the transcriptional activity of TR (24), it will be interesting to determine whether the properties of this mutant receptor reflect its altered interaction with such cofactors.

Acknowledgements

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E M and M G have contributed equally to this paper.

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