Role of leucine 341 in thyroid hormone receptor β revealed by a novel mutation causing thyroid hormone resistance

Karn Wejaphikul[1,2] MD, Stefan Groeneweg[1] MD, Prapai Dejkhamron[2] MD, Kevalee Unachak[2] MD, W. Edward Visser[1,3] MD, PhD, V. Krishna Chatterjee[3] MD, Theo J. Visser[1] PhD, Marcel E. Meima[1] PhD, Robin P. Peeters[1*] MD, PhD

[1]Department of Internal Medicine, Academic Center for Thyroid Diseases, Erasmus Medical Center Rotterdam, the Netherlands; [2]Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand; [3]Wellcome-MRC Institute of Metabolic Science, University of Cambridge, United Kingdom

E-MAIL ADDRESSES

k.wejaphikul@erasmusmc.nl, s.groeneweg@erasmusmc.nl, p.dejkhamron@gmail.com, kunachak@gmail.com, w.e.visser@erasmusmc.nl, kkc1@medschl.cam.ac.uk, t.j.visser@erasmusmc.nl, m.meima@erasmusmc.nl, r.peeters@erasmusmc.nl

ABBREVIATED TITLE: Role of leucine 341 residue in TRβ receptor

KEY TERMS: Resistance to Thyroid Hormone Beta; Thyroid Hormone Receptor Beta; Thyroid Hormone Action; Receptor Mutation

WORD COUNT: 750

NUMBER OF FIGURES: 1

Abstract

Leu341 has been predicted from crystal structure as an important residue for thyroid hormone receptor (TR) β function, but this has never been confirmed in functional studies. Here, we describe a novel p.L341V mutation as a cause of resistance to thyroid hormone β, suggesting an important role for Leu341 in TRβ function. *In silico* and *in vitro* studies confirmed that substituting Leu341 with Val and other non-polar amino acids impairs sensitivity of TRβ for T3 with various degrees, depending on their side-chain size and orientation.

Introduction

Mutations in the *THRB* gene that affect the function of the thyroid hormone receptor (TR) β cause resistance to thyroid hormone (RTH) β. The biochemical characteristics are elevated T4 and T3 with non-suppressed TSH concentrations. Based on the TRβ1 crystal structure, Leu341 has been predicted as an important residue for the binding to T3 (1, 2). We here verify the crucial role of Leu341 in T3 binding and TRβ1 functions, driven by the identification of a novel p.L341V mutation in an RTHβ patient.

Patient

A 12-year-old Thai girl (II.3) presented with short stature (height 134 cm [-3.17 SDS], weight 27.2 kg, BMI 15.1 kg/m^2 [-1.83 SDS]), diffused goiter and palpitations (heart rate 144/min). She had been misdiagnosed with Graves' disease and treated with methimazole for 3 years. During treatment, she had fluctuating thyroid hormone and increased TSH concentrations. Her older sister (II.2) and mother (I.2) also suffered from presumed Graves' disease, for which the mother had undergone a subtotal thyroidectomy and subsequently developed post-operative hypothyroidism which required high dose of L-thyroxine (300 µg/day) treatment. Their thyroid function tests showed high T4 and T3 with non-suppressed TSH concentrations, suggesting RTHβ (Fig.1A and Supplementary Fig.S1).

Results

After obtaining informed consent, sequencing of exons 7-10 of the *THRB* gene identified a novel heterozygous p.L341V (c.1021C>G) mutation in all affected members. This mutation is not present in public databases (dbSNP, 1000 Genomes, and Exome Aggregation Consortium [ExAC]).

Based on the crystal structure (PDB-ID: 3GWS (3)), Leu341 is located in the T3-binding pocket of wild-type (WT) TRβ1, and its aliphatic side-chain forms hydrophobic interactions with the outer ring of the T3 molecule and surrounding residues to maintain the shape and integrity of the T3-binding pocket (Fig.1B and Supplementary Fig.S2). TRβ1-L341V and three artificial mutants (L341I, L341A, and L341F) with hydrophobic side-chains of different sizes and structural properties were subsequently modeled (Fig.1B). Because the side-chain of Val is shorter than that of Leu and has a different orientation, the interaction with T3 and surrounding residues of TRβ1-L341V was predicted to be disturbed. Given its very small side-chain, these alterations were even more pronounced in the Ala substituent. Even though the size and branched-chain character of Ile is similar to Leu, the altered side-chain orientation affects direct contacts with T3 and interactions with the surrounding residues in TRβ1-L341I. Although TRβ1-L341F was predicted to slightly alter the architecture of the T3-binding pocket, the direct interactions with T3 and most of the surrounding residues were preserved.

In vitro studies confirmed the functional impairment of these mutants. In [^{125}I]T3 competitive binding assays, the dissociation constant (Kd) of all mutants was higher than WT, indicating a reduced T3 affinity. Interestingly, the shift in Kd was related to the size of the introduced side-chain and the distance to T3 and surrounding residues (Fig.1D). Substitution by Ala and Val, which have a smaller side-chain size than Leu, Ile and Phe, produced larger shifts in Kd. The shift of T3-induced transcriptional activity of the mutant receptors on the DR4-TRE luciferase reporter showed a similar trend. The half maximal effective T3 concentration (EC_{50}) of all mutants was higher than that of WT, indicating their impaired transcriptional activity (Fig.1E). In addition, the degree of the shift in EC_{50} also depended on the size and orientation of the side-chain. The EC_{50} of co-expressed WT and TRβ1-L341V was also significantly higher (3-

fold) than that of WT only, suggesting a dominant-negative effect of the TRβ1-L341V (Fig.1C). Together, these *in vitro* studies support an important role for Leu341 in T3 binding and receptor function.

Discussion

Here, we demonstrate that Leu341 of TRβ is crucial for T3 binding, prompted by the identification of a novel L341V *THRB* mutation in an RTHβ family. Our in-depth functional studies confirm the crucial role of this residue for TRβ function which had been predicted by crystallographic studies and the identification of a previously reported L341P mutation in a patient with RTHβ (4).

The *in silico* models used in this study correctly predict the degree of receptor impairment as found in our *in vitro* studies. In addition, the creation of artificial mutations based on the *in silico* modeling gains more detailed insight about the T3-TRβ interaction. It suggests that the exact side-chain size and orientation at residue 341 are of vital importance for T3 binding and hence receptor activity. These findings also indicate that the *in silico* prediction is a good approach to further explore the role of certain residues in TRβ function and may enhance our understanding of the pathogenic effects of mutations therein.

Acknowledgements

This work is supported by Zon-MWTOP Grant 91212044 and an Erasmus MC Medical Research Advisory Committee (MRACE) grant (RPP, MEM), Chiang Mai University (KW), and NIHR Cambridge Biomedical Centre (VKC).

Author Disclosure Statement

The authors have nothing to disclose.

Corresponding author and person to whom requests should be addressed:

Name: Robin P. Peeters, MD, PhD

Address: Academic Center for Thyroid Diseases, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands

Email: r.peeters@erasmusmc.nl

References

1. Li F, Xie Q, Li X, Li N, Chi P, Chen J, Wang Z, Hao C 2010 Hormone activity of hydroxylated polybrominated diphenyl ethers on human thyroid receptor-beta: in vitro and in silico investigations. Environ Health Perspect **118**:602-606.

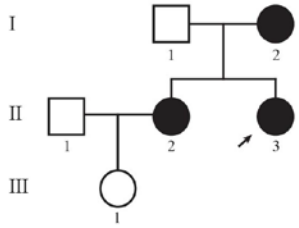
2. Hangeland JJ, Friends TJ, Doweyko AM, Mellstrom K, Sandberg J, Grynfarb M, Ryono DE 2005 A new class of high affinity thyromimetics containing a phenyl-naphthylene core. Bioorg Med Chem Lett **15**:4579-4584.

3. Nascimento AS, Dias SM, Nunes FM, Aparicio R, Ambrosio AL, Bleicher L, Figueira AC, Santos MA, de Oliveira Neto M, Fischer H, Togashi M, Craievich AF, Garratt RC, Baxter JD, Webb P, Polikarpov I 2006 Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. J Mol Biol **360**:586-598.

4. Rivolta CM, Olcese MC, Belforte FS, Chiesa A, Gruneiro-Papendieck L, Iorcansky S, Herzovich V, Cassorla F, Gauna A, Gonzalez-Sarmiento R, Targovnik HM 2009 Genotyping of resistance to thyroid hormone in South American population. Identification of seven novel missense mutations in the human thyroid hormone receptor beta gene. Mol Cell Probes **23**:148-153.

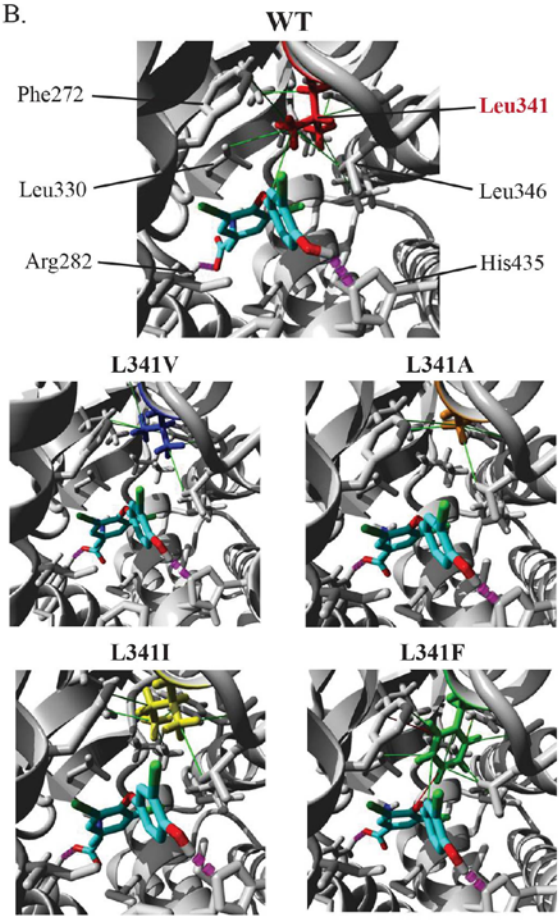
Figure 1. (A) The pedigree demonstrates three RTHβ patients in the family and their TFTs (TSH, thyroid-stimulating hormone; TT4, total thyroxine; FT4, free thyroxine; TT3, total triiodothyronine; FT3, free triiodothyronine; Anti-TPO, anti-thyroid peroxidase; Anti-TG, anti-thyroglobulin; TRAb, thyrotropin receptor autoantibody). (B) Crystal structure of T3-bound WT TRβ1 (PDB-ID: 3GWS) in which the side chain of the affected Leu341 is depicted in red. Arg282 and His435 form hydrogen bonds with the carboxyl group of the alanine side-chain and phenolhydroxyl group of T3, respectively (purple dashed lines). Together with Leu330, Phe272, and Leu346, Leu341 forms a hydrophobic pocket accommodating the two phenolic rings of the T3 molecule through hydrophobic interactions (green lines) which it stabilizes the hydrophobic pocket. Structural models of the L341V and three artificial mutants (L341A, L341I, and L341F) showing the side-chain size and orientation toward the T3 molecule and surrounding residues of the different residue side-chains. All mutants were predicted to disturb these interactions to various degrees, with the L341F having the smallest impact. (C) Co-transfection of WT with TRβ1-L341V alters transcriptional activity of WT in a dominant-negative manner (mean ± SEM of four independent experiments performed in triplicate). (D) The [^{125}I]T3 dissociation curves show the diverse severity of T3 binding impairment of the mutants (mean ± SEM of three independent experiments performed in duplicate). (E) Transcriptional activity of the mutant receptors is impaired, as indicated by the right-shifted of T3-induced dose-response curves tested on DR4-TRE (mean ± SEM of three independent experiments performed in triplicate). (Insert) Immunoblotting confirms the expression of all receptor constructs in Jeg3 cells.

A.

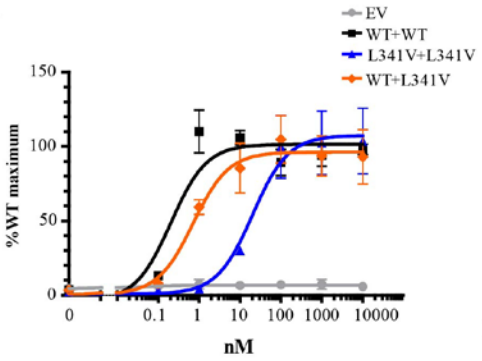


TFTs [normal range]	II.3	II.2	I.2
TSH (μIU/mL) [0.5-4.8]	3.29	2.45	1.2
TT4 (mg/dL) [4.2-13.0]	-	13.5	12
FT4 (ng/dL) [0.8-2.3]	5.37	-	-
TT3 (μg/dL) [55-170]	-	221	190
FT3 (pg/mL) [2.3-4.2]	14.31	-	-
Anti-TPO (IU/mL) [<40]	5.0	-	-
Anti-TG (IU/mL) [<125]	19.9	-	-
TRAb (IU/L) [0.00-1.75]	<0.3	<0.3	-

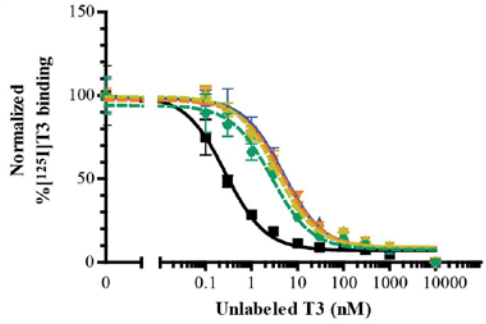
B.



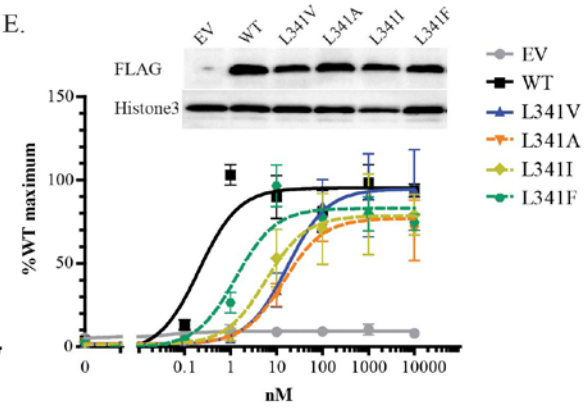
C.



D.



E.



Parameter	WT	L341V	L341A	L341I	L341F	p-value (One-way ANOVA[a])
LogKd [Kd(nM)]	-0.51±0.13 [0.31]	0.70±0.06** [5.01]	0.66±0.06** [4.54]	0.47±0.10** [2.94]	0.53±0.11** [3.42]	0.2606
$LogEC_{50}$ [EC_{50}(nM)]	-0.47±0.03 [0.34]	1.33±0.04*** [21.38]	1.37±0.04*** [23.12]	1.19±0.35*** [15.35]	0.33±0.06*** [2.14]	0.0113

Kd, dissociation constant; EC50, half maximal effective T3 concentration (student's t-test compared to WT, **p<0.01, ***p<0.001)
[a]One-way ANOVA compared between mutants

Supplementary Materials

Assessment of thyroid function

Thyroid function tests (TSH, FT4, and FT3) of the index patient were evaluated using electro-chemiluminescence immunoassay kit (Roche Diagnostic, Mannheim, Germany).

DNA extraction and mutation analysis

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). Exons 7-10 of the *THRB* gene [GeneBank: NM_000461.4], including exon-intron boundaries, were amplified (see Supplementary Table S1 for primers). Sequencing was performed as described previously (1). The exon carrying the mutation was re-amplified and sequenced to exclude a PCR error. The study was approved by the Medical Ethics Committee of the Faculty of Medicine, Chiang Mai University, Thailand.

In silico prediction of mutant TRβ1 function

YASARA Structure Software (YASARA Bioscience GmbH, Vienna, Austria) (2) was used to model the TRβ1-L341V patient's mutation and three artificial mutants (L341A, L341I and L341F) into a T3-bound WT TRβ1 crystal structure (PDB-ID: 3GWS) (3) using the side-chain substitution tool. Side-chain orientations were optimized using SCWALL (Side-Chain conformations With ALL available methods) (4, 5) after which the final models were minimized without further constraints. All images were created using YASARA Structure and Pov-Ray v3.6 software (www.povray.org).

DNA constructs and mutagenesis

The human TRβ1 cDNA was amplified and subcloned into the *EcoRI* and *XbaI* sites of the pcDNA3 expression vector fused at the 5'-end to the sequence encoding the FLAG-epitope

tag and downstream of an optimized Kozak sequence (see Supplementary Table S1 for primers). The TRβ1-L341V patient's mutation (c.1021C>G) and three artificial mutants, including L341A, L341I and L341F, were introduced using the QuickChange II Mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands) according to manufacturers' protocol (see Supplementary Table S1 for primers). Sequences of mutant constructs were confirmed by Sanger sequencing.

[^{125}I]T3 competitive binding assay

Human FLAG-tagged TRβ1 WT and mutant (L341V, L341A, L341I and L341F) receptor proteins were synthesized in reticulocyte lysate using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Leiden, The Netherlands). The protein lysate was incubated with 0.02 nM of [^{125}I]T3 in 0.5 mL binding buffer (20 mM Tris, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 5 mM DTT) and 0-10,000 nM unlabeled T3 for 2 hours at 30°C. Protein-bound [^{125}I]T3 was captured by filtering through a nitrocellulose filter membrane (Millipore HA filters, 0.45 µm) under vacuum. The data was corrected for non-specific binding (counts bound at 10,000 nM unlabeled T3) and expressed as percentage maximal [^{125}I]T3 binding (counts bound at 0 nM unlabeled T3). The [^{125}I]T3 displacement curve and the dissociation constant (Kd) were computed by GraphPad Prism program version 5.0 (GraphPad, La Jolla, CA) and shown as mean ± standard error of the mean (SEM) of three independent experiments performed in duplicate.

Cell culture and transfection

JEG3 cells were cultured and transfected as previously described (6). In brief, 20 ng of FLAG-tagged WT or mutant TRβ1 expression vectors and 120 ng of luciferase reporter constructs containing direct repeat (DR4) thyroid hormone response element (TRE) (7), as well

as 60 ng pMaxGFP transfection control, were transiently transfected into cells in thyroid hormone-depleted medium using Xtreme Gene 9 transfection reagent (Roche Diagnostics, Almere, NL). To determine the effect of TRβ1-L341V on WT function (dominant-negative effect), we co-expressed WT and TRβ1-L341V receptors (1:1 equimolar ratio), or either WT or TRβ1-L341V with empty vector (EV) (as gene dose control). After 24 hours, cells used for luciferase assays were incubated in DMEM/F12 medium supplemented with 0.1% bovine serum albumin and containing 0-10,000 nM T3 for 24 hours.

Immunoblotting

To determine the expression of FLAG-tagged TRβ1 WT and mutants in JEG3 cells, nuclear proteins were extracted as described previously with slight modifications (8). Briefly, cells were swollen on ice for 15 min in buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, pH 7.9) supplemented with the Complete Protease Inhibitor cocktail (Roche Diagnostics) and were lysed by addition of 0.6% NP40. The nuclei were pelleted by centrifugation for 10 min at 2500 *g* and extracted for 45 min in buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, Complete Protease Inhibitors, pH 7.9) at 4˚C. After centrifugation for 15 min at 20000 *g*, the supernatants containing nuclear proteins were collected and diluted in buffer D (20 mM HEPES, 1 mM EDTA, Complete Protease Inhibitors, pH 7.9). Immunoblotting was performed as previously described (6). The FLAG-tagged TRβ1 and Histone 3 (as loading control) were detected by FLAG-M2 antibody (#F1804 Sigma-Aldrich) and Histone 3 (H3; 1B1B2) antibody (#14269 Cell Signaling Technology), respectively, at a 1:1000 dilution and visualized by Enhanced Chemiluminescence (Thermofisher Scientific) on the Alliance 4.0 Uvitec platform (Uvitec Ltd).

Luciferase assay

Luciferase activity of WT and mutant receptors was measured using the Dual Glo Luciferase kit (Promega, Leiden, The Netherlands) as previously described (1). The ratio between luciferase and GFP was calculated to adjust for transfection efficiency. Data were expressed as percentage maximal response of WT and half maximal effective T3 concentration (EC_{50}) and maximal response calculated using GraphPad Prism program version 5.0 (GraphPad, La Jolla, CA). The results are shown as mean ± SEM of at least three independent experiments performed in triplicate.

References

1. van Mullem A, van Heerebeek R, Chrysis D, Visser E, Medici M, Andrikoula M, Tsatsoulis A, Peeters R, Visser TJ 2012 Clinical phenotype and mutant TRalpha1. N Engl J Med 366:1451-1453.
2. Krieger E, Vriend G 2014 YASARA View - molecular graphics for all devices - from smartphones to workstations. Bioinformatics 30:2981-2982.
3. Nascimento AS, Dias SM, Nunes FM, Aparicio R, Ambrosio AL, Bleicher L, Figueira AC, Santos MA, de Oliveira Neto M, Fischer H, Togashi M, Craievich AF, Garratt RC, Baxter JD, Webb P, Polikarpov I 2006 Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. J Mol Biol 360:586-598.
4. Canutescu AA, Shelenkov AA, Dunbrack RL, Jr. 2003 A graph-theory algorithm for rapid protein side-chain prediction. Protein Sci 12:2001-2014.

5. Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K 2009 Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. Proteins 77 Suppl 9:114-122.

6. van Gucht AL, Meima ME, Zwaveling-Soonawala N, Visser WE, Fliers E, Wennink JM, Henny C, Visser TJ, Peeters RP, van Trotsenburg AS 2016 Resistance to Thyroid Hormone Alpha in an 18-Month-Old Girl: Clinical, Therapeutic, and Molecular Characteristics. Thyroid 26:338-346.

7. Collingwood TN, Adams M, Tone Y, Chatterjee VK 1994 Spectrum of transcriptional, dimerization, and dominant negative properties of twenty different mutant thyroid hormone beta-receptors in thyroid hormone resistance syndrome. Mol Endocrinol 8:1262-1277.

8. Fozzatti L, Lu C, Kim DW, Cheng SY 2011 Differential recruitment of nuclear coregulators directs the isoform-dependent action of mutant thyroid hormone receptors. Mol Endocrinol 25:908-921.