A newly discovered TSHR variant (L665F) associated with non-autoimmune hyperthyroidism in an Austrian family induces constitutive TSHR activation by steric repulsion between TM1 and TM7

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Objective: New in-vivo mutations in G-protein coupled receptors open opportunities for insights into the mechanism of receptor activation. Here we describe the molecular mechanism of constitutive thyrotropin receptor (TSHR) activation in an Austrian family with three generations of familial non-autoimmune hyperthyroidism.

Patients: The index patient was diagnosed with hyperthyroidism during her first pregnancy. Her first two children were diagnosed with hyperthyroidism at the age of 11 and 10, respectively. TSH suppression was also observed in the third child at the age of 8, who has normal fT4 levels until now. TSH suppression in infancy was observed in the fourth child. The mother of the index patient was diagnosed with toxic multinodular goiter at the age of 36.

Methods: DNA was extracted from blood samples from the index patient, her mother and her four children. Screening for TSHR mutations was performed by high resolution melting assays and subsequent sequencing. Elucidation of the underlying mechanism of TSHR activation was carried out by generation and structural analysis of a TSHR transmembrane homology models and verification of model predictions by functional characterization of receptor mutations.

Results and conclusions: A newly discovered TSHR mutation L665F in transmembrane helix (TM) 7 of the receptor was detected in six members of this family. Functional characterization of L665F revealed constitutive activation for the Gs pathway and thus represents the molecular cause for hyperthyroidism in this family. The constitutive activation is possibly linked to a steric clash introduced by the L665F mutation between TMs 1, 2 and 7.

Thyrotropin (TSH) / TSH receptor (TSHR) signaling controls nearly every metabolic function in thyroid cells (1). After binding of TSH, the TSHR activates the Gs protein leading to an increase of intracellular cAMP in thyrocytes. As a direct result thyroid follicular cells grow and release thyroid hormones (1). At higher TSH concentration the receptor initiates the release of $G_{\alpha}q$, which subsequently leads to iodination and thyroid hormone synthesis (1–3).

Gain-of-function mutations in the TSHR gene are the major molecular cause for nonautoimmune hyperthyroidism (3). Gain-of-function mutations can be either somatic or germline. Somatic mutations are found in 60%–70% of hot thyroid nodules (4, 5). They lead to clonal expansion of the affected thyroid cell (6). The rare forms of sporadic nonautoimmune hyperthyroidism (SNAH, 16 published cases) or familial nonautoimmune hyperthyroidism (FNAH, 28 published cases) are mediated by germline mu-

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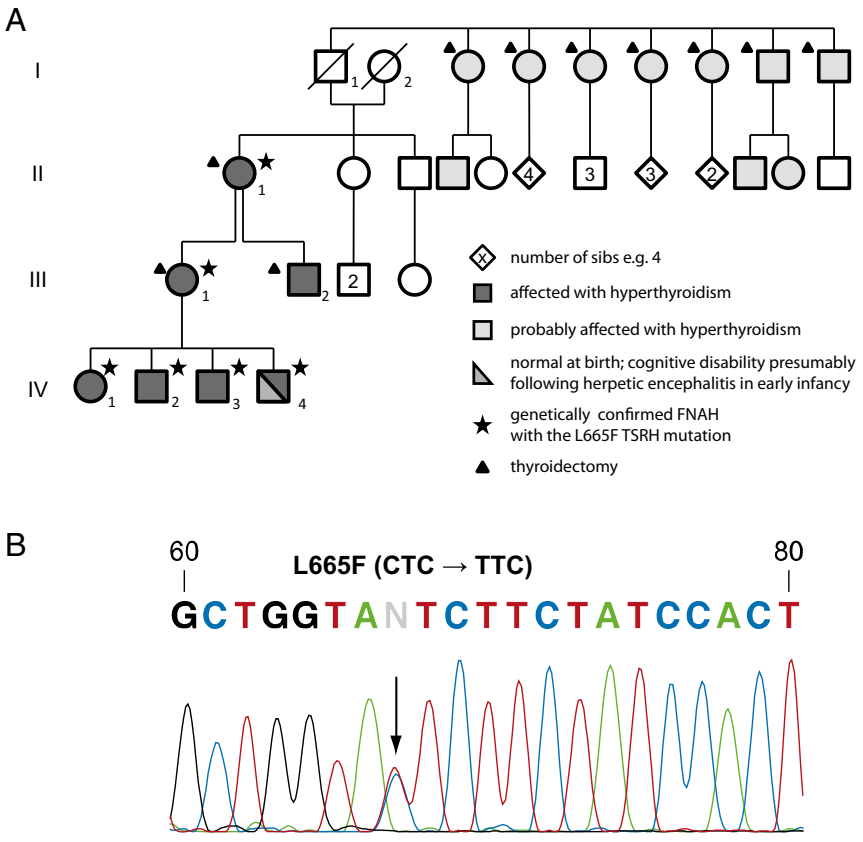

Figure 1. A) Pedigree of the Austrian family over four generations (I-IV). B) Result of one representative sequencing result for the Austrian family. All mutation positive family members have the same substitution (C to T) at 2149, which leads to an amino acid exchange (leucine to phenylalanine) at position 665 in the TSHR.

tations of the TSHR gene (7) (www.tsh-receptor-mutation-database.org). In families with FNAH the manifestation of hyperthyroidism can vary from 18 months to 74 years (8, 9). There is no relationship between the extent of basal TSHR activity and the clinical course of hyperthyroidism (10). However, the functional in vitro characterization of constitutively activating mutations (CAMs) found in vivo is a useful tool to understand the TSHR structure, function and activation process (11). This approach has also been applied to other G protein coupled receptors for which mutations have been detected in different diseases (12, 13).

CAMs cause a shift of the equilibrium from the inactive to an activated conformation typically induced and stabilized by binding of the endogenous ligand. Depending on the nature of the perturbation in the equilibrium varying degrees of basal activity are observed for different CAMs. The extent of the basal activity of CAMs is an important functional readout, which can help to identify structural features such as residue interactions necessary for a proper receptor function (14–18). Yet not only the identification and characterization of specific CAMs but also information about shared mechanisms and links of the respective constitutively activating mutation with other CAMs can therefore be crucial for understanding the mechanism of activation of the TSHR and related receptors.

Patients, Materials and Methods

Patient

The index patient (III.1 see Figure 1A) was diagnosed with hyperthyroidism (increased fT4 and fT3 and suppressed TSH at presentation) with undetectable TPO and TSHR antibodies aged 18 years during her first pregnancy. After two years of treatment with thiamazole her fT4 increased to > to 40.94 pmol/L. Therefore she underwent thyroid surgery at the age of 22 years. Her mother (II,1) was also treated for several years for hyperthyroidism (increased fT4 and fT3 and suppressed TSH at presentation) and eventually underwent surgery at the age of 36 years. Similarly, thyroid surgery was also performed in the half-brother (III,2) for toxic nodular goiter at the age of 15 years (increased fT4 and fT3 and suppressed TSH at presentation) after relapse of hyperthyroidism during antithyroid treatment. The maternal grandfather died of cardiac failure, thyroid disease was not reported and no thyroid hormone determinations are available. All seven siblings of the grandfather (I.1) underwent surgery for nodular goiter, unfortunately records of lab results are no longer available to confirm the likely hyperthyroidism for these seven siblings. Only a partial family history (but no medical records) could be obtained for some of the descendants of these seven siblings. At least 3 offspring of these siblings underwent thyroidectomy. No thyroid disorder or treatment was noticed in the mother of II,2.

The index patient (III.1) gave birth to 4 children. Of these the older ones (IV.1,2) were diagnosed with hyperthyroidism and slightly enlarged thyroids at 10 and 11 years, respectively. Thiamazol therapy led to normal T4 levels. TSH suppression was also found in the third child at the age of 8 years (IV.3). He still shows normal T4 levels and has not jet received antithyroid treatment up to present. The youngest boy (IV.4) exhibited TSH suppression (<0.01 mU/l, fT4 with 1.37 ng/dl still normal) already at 6 months and was started on thiamazol because of hyperthyroidism at the age of 2 years. Sadly, his development is

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impaired due to person affected by epilepsy encephalopathy following a herpes simplex stomatitis in infancy (before the onset of hyperthyroidism). Currently all thiamazol-treated children show normal fT4 and fT3 values during antithyroid drug treatment for 3 (IV.1), 2 (IV.2), 1 (IV.3) and 1 1/2 (IV.4) years. A follow up of their available TSH values during their ongoing antithyroid drug treatment is summarized in supplemental Table 1 and the most recent thyroid parameters in supplemental Table 2. Unfortunately, the father of these children was not available for a medical history or for laboratory results.

Nucleic acid isolation and detection of point mutations by high resolution melting (HRM) peak analysis

Genomic DNA was extracted from peripheral blood leukocytes (Qiagen Blood Kit, Qiagen, Chatsworth, CA) obtained from the index patient (mother), her four children, and her grand mother. The screening for TSHR mutations in exons 9 and 10 was performed by real time PCR and high resolution melting (HRM) peak analysis, using the primers shown in supplemental Table 3 and the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche, Mannheim, Germany). PCRs to detect TSHR point mutations were processed through an initial denaturation at 95°C for 10 minutes followed by 55 cycles of a 3-step PCR, including 3 seconds of denaturation at 95°C, a 10 seconds annealing phase at 56°C, and an elongation phase at 72°C for 10 seconds. Hereafter, a high resolution melting curve was assessed from 75°C to 95°C with an increase of 0.02°C/sec and 25 acquisitions per degree.

DNA from patient specimens known to carry a TSHR point mutation were used as positive controls in each analysis. Samples tested positive were subsequently sequenced using Big Dye-terminator chemistry (Applied Biosystems, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3100 (Applied Biosystems, Germany).

Site-directed Mutagenesis

cDNA for human TSHR was inserted into the pcDNA3.1(-)/hygromycin vector using restriction sites *XhoI* and *BamHI*. Mutations were introduced into hTSHR-pcDNA3.1 via site-directed mutagenesis, as described previously (19), using specifically designed primers. Mutated TSHR sequences were verified by Big Dye-terminator chemistry (Applied Biosystems, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3100 (Applied Biosystems, Germany).

Cell culture and transient expression of mutant TSHR

COS-7 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) supplemented with 10% FCS, 10^5 U/l penicillin and 100 mg/l streptomycin (Gibco Life technologies, Paisley, UK) at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected in 12-well plates (1×10^5 cells per well for COS-7) or 24-well plates (0.5×10^5 cells per well for COS-7) with 1 μg and 0.5 μg DNA per well, respectively, using the GeneJammer® Transfection Reagent (Stratagene, Amsterdam, NL).

FACS analyses

Transfected cells were detached from the dishes with PBS containing 0.1% BSA (FACS buffer) and transferred into Falcon 2054 tubes. Cells were washed once with FACS buffer and then incubated at 4°C for 30 minutes with a 1: 400 dilution of a mouse anti human TSHR antibody (2C11, 10 mg/l, Serotec Ltd., Oxford, UK) in the same buffer. Cells were washed twice and incubated at 4°C for 30 minutes with a 1 : 400 dilution of an Alexa Fluor 488-labeled $F(ab')_2$ fragment of goat antimouse IgG (Invitrogen, Molecular Probes, Eugene, Oregon, USA) in FACS buffer. Before FACS analysis (FACscan Becton Dickinson and Co., Franklin Lakes, NJ, USA) cells were washed twice and then fixed with 1% paraformaldehyde. Receptor expression was determined by the median fluorescence intensity (MFI). The TSHR was set at 100% and receptor expression of the mutant was calculated according to this. The percentage of signal positive cells corresponds to transfection efficiency, which was approximately 50%–60% of viable cells.

cAMP accumulation assay

For cAMP assays cells were grown and transfected in 24-well plates. Forty-eight hours after transfection, cells were incubated in the absence or presence of 100 mU/ml rhTSH in serum free medium supplemented with 1 mM IBMX (Sigma) for one hour. Recombinant human TSH (Thyrogen) was purchased from Genzyme (Neu-Isenheim, Germany). Reactions were terminated by aspiration of the medium. The cells were washed once with ice cold PBS and then lysed by addition of 0.1 N HCl. Supernatants were collected and dried. cAMP content of the cell extracts was determined using the cAMP AlphaScreen™ Assay (PerkinElmer) according to the manufacturer's instructions.

Linear regression analyses of constitutive activity as a function of TSHR expression (slopes)

Constitutive activity is expressed as basal cAMP formation as a function of receptor expression determined by

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FACS. COS-7 cells were transiently transfected in 24-well plates with increasing concentrations of wt or mutant TSHR plasmid DNA (50; 100; 200; 300; 400; and 500 ng per well). To transfect a constant DNA amount of 500 ng empty vector plasmid DNA was added to the mutant or wt DNA. For determination of cell surface expression see "FACS analyses". For determination of basal intracellular cAMP accumulation see "cAMP accumulation assay". Basal cAMP formation as a function of receptor expression was analyzed using the linear regression module of GraphPad Prism 4 for Windows.

Activation of inositol phosphate (IP) formation

Forty eight hours after transfection of COS-7 cells in 12 well plates, cells were incubated with 2 μCi [myo-^{3}H]inositol (Amersham Biosciences, Braunschweig, Germany) for 6 hours. Thereafter, cells were incubated with serum-free DMEM containing 10 mM LiCl and 100 mU rhTSH/ml for stimulation. Recombinant human TSH (Thyrogen) was purchased from Genzyme (Neu-Isenheim, Germany). Evaluation of basal and rhTSH-induced increases in intracellular IP levels was performed by anion exchange chromatography as previously described (20). Inositol phosphate (IP)-values were expressed as the percentage of radioactivity incorporated from [^{3}H]IP-1 to –3 over the sum of radioactivity incorporated in IPs and phophatidylinositol (PI).

Homology model of the TSHR

For structural examination a homology model of the hTSHR transmembrane domain was generated using Rosetta 3 (21) as described by Dong et al (22). Briefly, the protein sequence of the hTSHR was aligned to the structural coordinates of each of 19 distinct template structures (see supplemental Table 4). For each template 200 models were built reconstructing backbone coordinates in gapped regions of the alignment using the cyclic coordinate descent (CCD) protocol. The lowest energy model for each template was chosen for a more extensive sampling of the conformation of extracellular loops: 1000 models were built for each template reconstructing the loop regions with the CCD protocol. In all instances side chain coordinates were added from a rotamer library. The 10% lowest energy structures were clustered using bcl::Cluster (23) with a cluster radius of 3.0 Å. For final analysis models were evaluated based on low energy and cluster size. In addition a contact map for the 10% lowest energy structures of each template was generated with Rosetta's contactMap protocol with a CB distance cutoff of 10 Å. Visualization and image generation was done using the PyMOL Molecular Graphics System (Version 1.5.0.4 Schrödinger, LLC).

Statistics

Statistical analysis was carried out using the nonparametric *t* test using GraphPad Prism 4 for Windows.

Results

Mutation analysis

To confirm the hypothesis of nonautoimmune congenital hyperthyroidism in the Austrian family we searched for TSHR mutations by HRM and subsequent sequencing using DNA extracted from the patients blood leukocytes. A new TSHR mutation was identified in family members (IV.1 – 4 and III.1–2 and II.1 - Figure 1A). The newly identified TSHR variant leads to a transition in position 2149 from C to T of the TSHR gene (Figure 1B). The mutation is heterozygous and results in a substitution of phenylalanine (TTC) for leucine (CTC) at amino acid residue 665.

Functional characterization of the new TSHR variant L665F

Functional characterization of mutation L665F revealed a cell surface expression comparable to the wt TSHR (Figure 2A). Determination of intracellular cAMP levels confirmed the hypothesis of a constitutively activating TSHR mutation as the molecular cause of nonautoimmune congenital hyperthyroidism. Mutation L665F showed a significant increase of the ligand independent basal cAMP level of 3 fold over the basal value of the wt TSHR and a slightly higher cAMP response after administration of rhTSH compared to the wt TSHR (Figure 2B). To further validate the constitutive activity of TSHR variant L665F we performed linear regression analyses (LRA), which allowed us to compare the receptors basal activity independently from the cell surface expression. The data obtained by LRA further confirmed the constitutive activity of the L665F variant by a slope of 3.0 (wt TSHR set at 1) (Figure 2C). Investigation of the mutation's capability to activate the Gq pathway did not show an altered basal activity or response to rhTSH (Figure 2D).

Close proximity of TSHR positions V421 (TM1) and L665 (TM7)

In the best scoring homology model, L665 is near three residues within TM1 and TM2, namely V421, A471 and L475. In particular the contact between L665 and V421 occurs with the highest frequency (93%) in the best scoring homology models (see supplemental Table 5). This is striking, as V421 is part of a cluster of amino acids between TM1 and 7 for which constitutively activating mutations are known (Figure 3A) (5, 16, 24–28). It has been suggested that Val421 (TM 1), Leu467 and A471 (TM 2)

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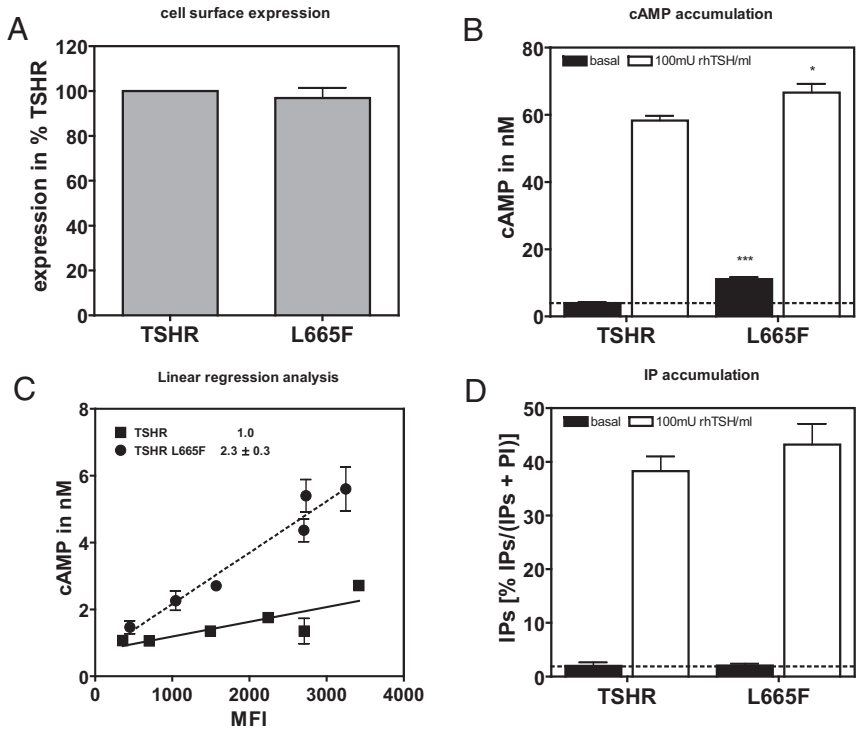

Figure 2. Functional characterization of TSHR mutation L665F. A) Cell surface expression of L665F in relation to the wt TSHR. MFI (mean fluorescencs intensity). B) Intracellular basal and TSH-mediated cAMP accumulation without normalization of the expression levels. C) Determination of the constitutive activity independently from the mutation's cell surface expression by linear regression analyses (LRA). D) Basal and TSH-mediated activation of the Gq pathway. Inositol phosphate (IP) -values were expressed as the percentage of radioactivity incorporated from [^{3}H]IP-1 to –3 over the sum of radioactivity incorporated in IPs and phophatidylinositol (PI). Data are presented as mean ± SD of at least three independent experiments, each performed in duplicate. *** $P < .001$, ** $P = .001–0.01$, * $P = .01–0.05$

as well as Leu665 (TM7) form a hydrophobic patch and functional characterization of V421I showed a similar phenotype like the new variant L665F (28). We hypothesize that V421I and L665F cause constitutive activation of the TSHR via the same mechanism. In both cases substitution of the side chain with a slightly larger variant resulted in constitutive activation of the TSHR, indicating that steric repulsions of the larger side chain with other parts of the protein push the receptor towards the activated conformation. The best models based on the inactive and active conformation of the A2A adenosine receptor (PDB ID: 3eml and 2ydv) support this theory by showing an increased distance between V421 and L665 in the active conformation (Figure 3B).

L665/V421 Double-mutant cycle analysis

To test whether the constitutive activity of L665F is at least partially caused by steric repulsion with V421, we generated several single and double mutants. Our strategy was to replace each amino acid with a smaller amino acid (V421A and L665V) and the larger CAM (V421I and L665F). Then we created double mutants combining the four mutations. Further we tested a V421L mutant to compare the effect of steric bulk vs steric hindrance in that position. All single and double mutations were well expressed with expression levels between 84%–99% when compared with the wt TSHR. The functional characterization of the generated constructs is summarized in Table 1.

Added steric bulk in position L665 and V421 stabilizes the active state of TSHR

Stimulation with 100 mU/ml rhTSH resulted for some of the TSHR variants (V421I, L665F, V421I/L665F) in slightly but significantly increased cAMP levels after administration of rhTSH (Table 1). V421L/L665F caused significantly increased, whereas V241A displays somewhat reduced ligand induced cAMP levels. It is striking that mutation to a smaller amino acid in V421A and L665V resulted in TSHR variants exhibiting ligand induced cAMP levels similar to or below the level of the wild type receptor. In contrast varying degrees of elevated cAMP levels after rhTSH stimulation, when compared with the wt TSHR, were only observed for single or double mutants containing one or two larger amino acids, an observation consistent with the notion that added steric bulk in this region stabilizes the receptor in an activate state even after ligand binding. However, overall these effects were small or moderate compared to the functional differences observed for the basal receptor state.

V421 and L665 form the center of a hydrophobic cluster critical for TSHR activation

The combination of the newly identified CAM L665F with the previously described CAM V421I (28) in the double mutant V421I/L665F resulted in an increased basal cAMP activity (LRA: 3.4) compared to the respective single mutants L665F (LRA: 3.0) and V421I (LRA: 2.0, Table 1, Figure 3C). Next we asked if mutation to a smaller amino acid in one location can compensate for mutation to a larger amino acid in the other position. This experiment tests if the direct interaction of L665 and V421 is the only cause for constitutive activity. In this case we would expect a wild type-like phenotype as the mutations should compensate each other.

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The combination of the CAM V421I with L665V to V421I/L665V displayed a basal activity similar to the native TSHR (Table 1, Figure 3C). Thus, in this scenario the substitution of the L665 side chain with the smaller V apparently compensates the V421I mutation. Furthermore, we generated the complementary exchange of V421 and L665 (V421L/L665V double mutant). While the single V421L exchange resulted in an elevated basal cAMP activity (LRA: 4.4) that even exceeded the basal activity of V421I, the L665V variant displayed a basal cAMP activity (LRA: 0.8) similar to the native TSHR (Table 1, Figure 3C). Here, the complementary exchange V421L/L665V displayed an elevated basal cAMP activity even slightly higher than the V421L single mutant (Table 1, Figure 3C).

To test whether substitution of V421 with a smaller amino acid could compensate the constitutive activity of the L665F mutation we mutated V421 to an A and created the V421A/L665F double mutant. The mutation V421A resulted in decreased basal activity (LRA: 0.4) of the TSHR. The combination with the CAM L665F resulted in a constitutively active V421A/L665F variant (LRA: 1.8) of the receptor, yet its constitutive activity is significantly decreased in comparison to the L665F single mutant (Table 1, Figure 3C).

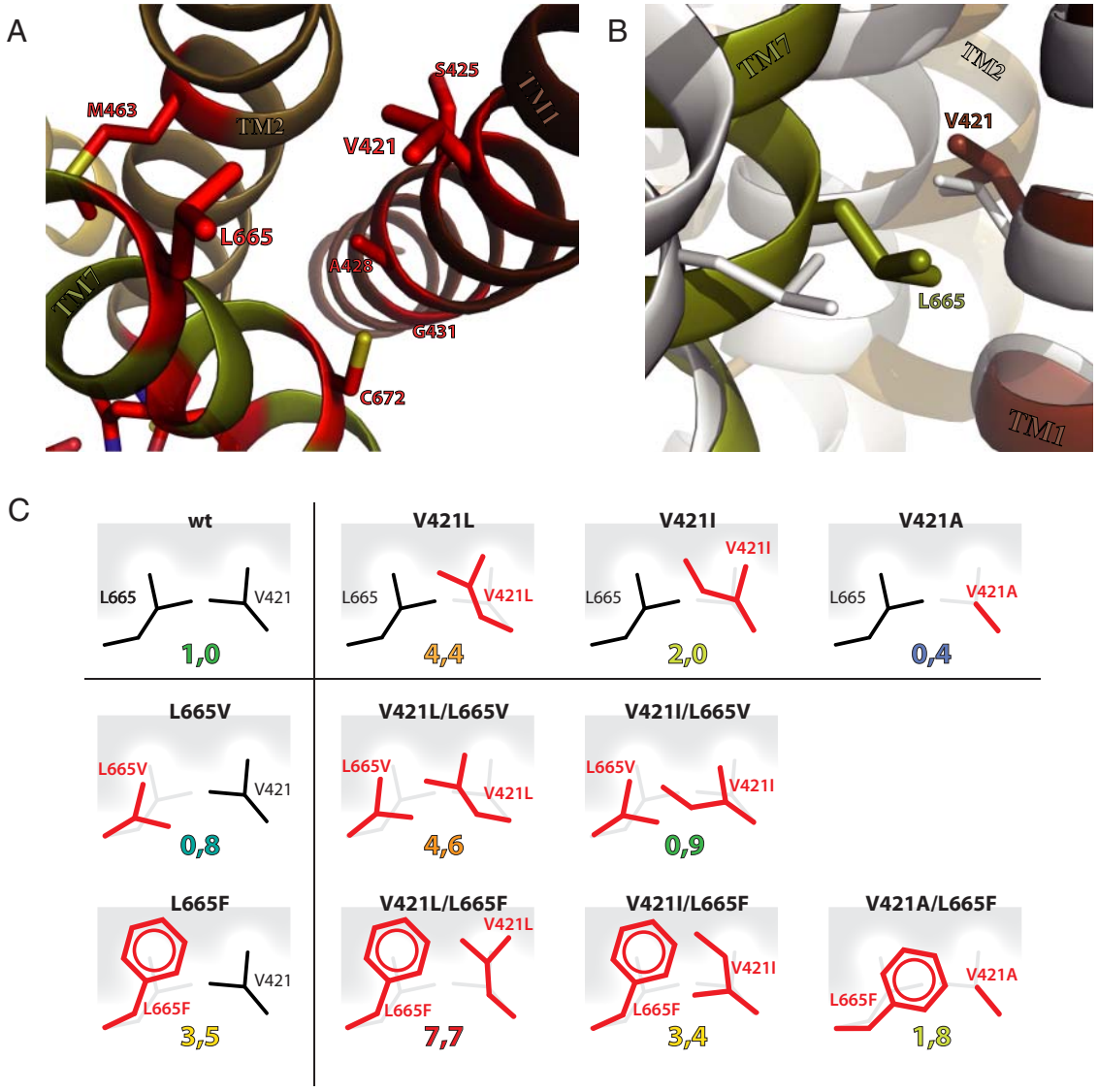

Figure 3. A) Lowest energy homology model of the hTSHR, which is based on the crystal structure of the rat M3 muscarinic acetylcholine receptor (PDB entry: 4daj). In this model amino acid V421 is part of a cluster of residues (highlighted in red) in TM1 and 7 for which constitutively activating mutations have been reported. B) Homology model of the hTSHR based on the crystal structure of the A2A adenosine receptor in its inactive (colored, PDB entry: 3eml) and active state (white, PDB entry: 2ydv) showing the side chains of L665 in TM7 and V421 in TM1. The model of the active state displays an increased distance between L665 and V421. C) Schematic representation of the generated TSHR single and double mutants and their slope determined by linear regression analyses (see also Material and Methods).

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Table 1. Functional characterization of TSHR single and double mutants.

Construct	Localization	Cell surface expression	cAMP accumulation (nM) basal	100mU/ml TSH	LRA (slope)
TSHR	-	**100**	**3.9 ± 0.3**	**52.7 ± 1.8**	**1**
pcDNA	-	**3 ± 1**	**1.6 ± 0.2**	**2.2 ± 0.3**	**-**
V421A	TM1	**93 ± 2***	**2.5 ± 0.1**	**43.8 ± 3.0**	**0.4 ± 0.1**
V421I	TM1	**96 ± 7**	**6.9 ± 0.5*****	**67.3 ± 2.9***	**1.5 ± 0.5**
V421 liter	TM1	**89 ± 5**	**17.4 ± 0.6*****	**62.8 ± 3.1**	**4.3 ± 0.5***
L665F	TM7	**97 ± 5**	**11.1 ± 0.6*****	**65.2 ± 3.6***	**2.3 ± 0.3***
L665V	TM7	**99 ± 4**	**2.2 ± 0.1*****	**55.8 ± 3.5**	**0.8 ± 0.1**
V421A/L665F	TM1/TM7	**99 ± 6**	**4.8 ± 0.2**	**51.8 ± 3.3**	**1.6 ± 0.2**
V421I/L665F	TM1/TM7	**92 ± 14**	**13.3 ± 0.5****	**68.7 ± 1.5****	**3.4 ± 0.3***
V421I/L665V	TM1/TM7	**98 ± 4**	**3.9 ± 0.5**	**60.4 ± 3.0**	**0.9 ± 0.0**
V421 liter/L665F	TM1/TM7	**88 ± 6**	**28.0 ± 1.3*****	**81.3 ± 4.1****	**7.7 ± 0.6***
V421 liter/L665V	TM1/TM7	**84 ± 4****	**13.2 ± 0.1*****	**58.0 ± 2.3**	**4.5 ± 0.7***

COS-7 cells were transfected with plasmids harboring the nucleotide sequence of wild type TSHR or various mutant TSHRs. The pcDNA3.1(-)/hygromycin was used as a control. Cell surface expression was quantified on a FACS flow cytometer. Basal and TSH-mediated levels of cAMP were determined after treatment of the cells with or without 100 mU/ml recombinant human TSH. Data are given as mean ± SD of at least three independent experiments ($n = 3$), each carried out in duplicates.

*** $P < 0.001$, ** $P = 0.001–0.01$, * $P = 0.01–0.05$

Discussion

The molecular cause for nonautoimmune hyperthyroidism in an Austrian family is due to TSHR mutation L665F

The L665F mutation is a newly discovered TSHR germline mutation (www.tsh-receptor-mutation-database-.org). Since the identification of TSHR mutations as molecular cause of familial nonautoimmune hyperthyroidism in 1994 after its first clinical description in 1982, 29 families with 20 different point mutations, including L665F, have been reported (25, 29). In this Austrian family hyperthyroidism without symptoms of auto-immune thyroid disease was diagnosed in three generations. Interestingly, all four children of the third generation are carriers of the TSHR mutation, which is an unusual but not impossible event for an autosomal dominant inheritance. As previously observed in other families (8, 10) the onset of hyperthyroidism does not correlate with the extent of the ligand independent basal activity as also shown by the different ages of onset of hyperthyroidism ranging from 2–11 years for the four children.

TSHR L665 is part of a cluster of hydrophobic amino acids in TM1 and 7 for which CAMs have been reported

For some of the 61 currently known mutations that constitutively activate the TSHR in vivo (www.tsh-receptor-mutation-database.org) the specific molecular mechanism of receptor activation has been identified. This provided useful information regarding the activation mechanism of the TSHR (14–18). Based on predictions deduced from a homology model of the hTSHR transmembrane domain, we performed in vitro experiments to decipher the molecular action of the newly identified L665F mutation. Structural analysis places L665 in a cluster of amino acids in TM1 and 7 (Figure 3A) for which CAMs have been reported. The exchange of the native L at position 665 with a larger F, as found in the Austrian family, results in an increased basal Gs activity of the TSHR. In all reported CAMs at the TM1/7 interface constitutive receptor activity is caused by substitutions of the initial residue by a residue with a larger side chain as shown for V421, S425, A428, G431 of TM1 and C672 of TM7 (Figure 3A) (5, 14, 24–28). This suggests that larger side chains at the respective residues push the receptor towards a ligand independent, activated conformation. This effect has been described also for a M626I mutation (30). However, in contrast to M626I the CAMs in TM1 and 7 are not near the interface of the TM domain with the G protein (31).

While the environment of L665 in the basal state of the TSHR is most likely composed of several amino acids we focused our analysis on establishing a connection between the constitutive activity of L665F and the previously reported V421I. An increase in basal activity mediated by a larger side chain was also observed for the newly generated single mutant V421L and the double mutants V421L/L665F and V421I/L665F. Furthermore, the effect of a longer or bulkier side chain at one position can be compensated by introducing a smaller side chain at a position in close proximity as shown for TSHR variants L665V, V421A, V421I/L665V and V421A/L665F. The observation that the introduction of the smaller V side chain at position L665 decreases the constitutive activity introduced by the V421I mutation as well as the reduction in constitutive activity of the V421A/L665F double mu-

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tant in comparison to the L665F single mutant supports the close spatial proximity of L665 (TM7) and V421 (TM1) as suggested by the homology model of the hTSHR transmembrane domain. Comparison of the results for variants V421I and V421L showed that not only the size of the side chain affects the extent of the constitutive activity but also the stereochemistry of the introduced amino acid. The extra methyl group of the I in the V421I mutation increases the basal activity only slightly when compared with the TSHR. In contrast, the additional alteration of the center of mass of the L side chain in the V421L substitution (Figure 3C) resulted in an even higher basal receptor activity due to an increased steric repulsion with adjacent amino acids. The shifted center of mass apparently also prevents the L side chain of the V421L mutant from relocating to the space freed by the L665V mutant resulting in an unchanged constitutive activity of the V421L/L665V double mutant in comparison to the V421L single mutant. These findings further indicate that next to the L665/V421 interaction, additional residues are involved in the constitutive activity of CAMs L665F, V421I and V421L.

Taken together, we investigated a family with FNAH in three generations and identified the new TSHR mutation L665F as the molecular cause for this particular thyroid disorder. Furthermore, by a detailed mutagenesis study, we showed that steric repulsion between TM1 and TM7 is most likely the mechanism by which mutation L665F, V421I and V421L, as well as the previously reported CAMs at the interface of TM1 and TM7 lead to constitutive activation of the TSHR.

Acknowledgments

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