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Mutations that silence constitutive signaling activity in the allosteric ligand-binding site of the thyrotropin receptor

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Abstract

The thyrotropin receptor (TSHR) exhibits elevated cAMP signaling in the basal state and becomes fully activated by thyrotropin. Previously we presented evidence that small-molecule ligands act allosterically within the transmembrane region in contrast to the orthosteric extra-cellular hormone-binding sites. Our goal in this study was to identify positions that surround the allosteric pocket and that are sensitive for inactivation of TSHR. Homology modeling combined with site-directed mutagenesis and functional characterization revealed seven mutants located in the allosteric binding site that led to a decrease of basal cAMP signaling activity. The majority of these silencing mutations, which constrain the TSHR in an inactive conformation, are found in two clusters when mapped onto the 3D structural model. We suggest that the amino acid positions identified herein are indicating locations where small-molecule antagonists, both neutral antagonists and inverse agonists, might interfere with active TSHR conformations.

Keywords

G-protein-coupled receptor (GPCR); Thyrotropin receptor; Signal transduction; Allosteric binding pocket; Antagonist; Silencing mutations

Introduction

The thyrotropin (thyroid-stimulating hormone, TSH) receptor (TSHR), the lutropin receptor (luteinizing hormone/choriogonadotropin receptor, LHCGR) and the follitropin receptor (follicle-stimulating hormone receptor, FSHR) are glycoprotein hormone receptors (GPHRs) that constitute subfamily 1C of the rhodopsin-like (family 1) G-protein-coupled receptors (GPCRs) with seven transmembrane helices (seven-transmembrane helix receptors, 7TMRs) [1]. The TSHR and the endogenous agonist TSH are pivotal proteins with respect to a variety of physiological functions including control of thyroid growth and regulation of metabolic cycles [2–4]. The TSHR is also known to be expressed in multiple extrathyroidal tissues, including bone, ovary, kidney, testis, and cells of the immune system [5–14], but the regulatory role of the TSHR in these tissues is not understood.

A functional feature of the TSHR is an elevated level of cAMP accumulation in the basal state (in absence of thyrotropin). Malfunction of the TSHR can cause non-autoimmune diseases or participate in autoimmune diseases characterized by a hyper- or hypo-functioning thyroid gland. Antibodies directed at TSHR and naturally occurring mutations in TSHR are determinants of several thyroid-malfunctions [15–26]. In general, such mutations and antibodies can be distinguished by either inactivating (loss-of-function, hypothyroidism) or activating (gain-of-function, hyperthyroidism) effects. Numerous pathogenic constitutively activating mutations (CAMs) that increase basal TSHR signaling independently from the hormone have been found [27]. The identification of antagonistic molecules that might be able to suppress constitutive activity is a special focus of TSHR research. Such compounds might have therapeutic potential for pharmacological intervention of TSHR-mediated diseases [24, 28, 29]. Several strategies to develop antagonists—both neutral antagonists that inhibit TSH or antibody-mediated activation of the TSHR, and inverse agonists that inhibit constitutive signaling by TSHR—for direct therapeutic intervention have been pursued (reviewed in [30–32]). Thyroid blocking antibodies (TBABs) have been shown to exhibit neutral antagonist or inverse agonist properties at TSHR [24, 28, 29]. Recently, the first low to moderate affinity antagonistic small-molecule ligands for the TSHR were identified [33, 34].

Approaches of combined site-directed mutagenesis studies and homology modeling of the TSHR have provided evidence that in contrast to the orthosteric hormone binding site, such drug-like small molecules bind allosterically in the transmembrane region [33, 35, 36]. We found points of interaction for TSHR agonists and antagonists. In particular, recent site-directed mutagenesis at residues surrounding the allosteric binding pocket led to constitutive TSHR activation [37]. These CAMs are indicators for signaling-sensitive points of activation and might also be potential interaction partners for small-molecule agonists to induce TSHR activation.

Here we hypothesized that the transmembrane region in close proximity to the small ligand binding site may also harbor amino acids which are sensitive for silencing effects on constitutive receptor activity. Indeed, we report the identification of several silencing mutations flanking the allosteric binding pocket. These amino acid positions might play an important role as points of small-molecule antagonist interactions that can inhibit TSHR signaling. Our long-term goal is to understand the diverse intramolecular events that occur

in or adjacent to the allosteric binding site. This knowledge will be important for the development of small-molecule ligands for pharmacological interventions at the TSHR. Based on the conservation of structural features and amino acid composition among the family A GPCRs, our study also provides interesting information regarding a ligand binding region that is of importance also for other receptors.

Materials and methods

Construction of vectors and site-directed mutagenesis

The restriction site *KpnI* was introduced into hTSHR-pSVL by use of QuikChange Site-Directed Mutagenesis Kit (Stratagene). Wild-type (wt) TSHR sequence was cloned into pcDNA3 vector (Invitrogen) via *KpnI* and *BamHI* restriction sites. Mutations were introduced into wt TSHR in vector pcDNA3 using QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sequences of all constructs were verified by nucleotide sequencing.

Cell culture and transfection

Wild-type and mutated TSHRs were expressed in HEK 293 cells (DSMZ). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Biocrom) at 37°C in a humidified 5% CO₂ incubator. For measurement of intracellular cAMP and IP accumulation, cells were seeded in 24-well plates (7.5 × 10⁴ cells per well) and transfected with 0.6 µg DNA/well. For determination of receptor expression at the cell surface, cells were seeded in six-well plates (3 × 10⁵ cells per well) and transfected with 2.4 µg DNA/well. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) 24 h after seeding.

Determination of cell surface expression by FACS

The expression levels of wt TSHR and TSHR mutants were quantified on a FACS flow cytometer (FACSCalibur, Becton–Dickinson). All steps were performed at 4°C or on ice. 48 h after transfection, cells were harvested using 1 mM EDTA and 1 mM EGTA in PBS. After detachment, cells were spun at 300 × g for 3 min and the supernatant was discarded. Cells were incubated for 15 min in FACS buffer (PBS containing 0.5% BSA), spun and subsequently, they were incubated for 30 min with a 1:200 dilution of a mouse anti-extracellular human TSHR antibody (2C11, Serotec) in FACS buffer. Cells were washed twice in FACS buffer and incubated for 30 min with a 1:400 dilution of a DyLight488-conjugated goat anti-mouse IgG antibody (Serotec). Subsequently, cells were washed twice and resuspended in FACS buffer. For exclusion of damaged cells from analysis, 7-AAD (Becton–Dickinson) was added. The fluorescence of at least 10,000 cells per tube was assayed. Receptor expression was determined by the mean fluorescence intensity by comparison to wt TSHR.

Determination of intracellular cyclic AMP accumulation by RIA

After transfection, cells were cultured for 48 h and stimulated for 1 h at 37°C with stimulation buffer (DMEM supplemented with 10 mM HEPES, 0.5% BSA, 0.25 mM 3-isobutyl-1-methylxanthine) alone or stimulation buffer containing increasing concentrations of bTSH (1 µIU/ml to 0.1 IU/ml, Sigma-Aldrich). Stimulation medium was aspirated and reactions were stopped by lysis of the cells with 0.1% trifluoroacetic acid, 0.005% Triton X-100 for 30 min at 4°C. Supernatants were collected and heated at 95°C for 10 min, dried in a rotation vacuum concentrator (Alpha-RCV, Christ) and finally stored at –20°C until use. After reconstitution and acetylation of the samples [38], the cAMP content was determined using cAMP-¹²⁵I-tyro-sylmethylester (10,000 cpm, IBL) and polyclonal rabbit anti cAMP-antibody (final dilution 1:1,60,000). Samples were incubated overnight at 4°C. The

antibody-bound fraction was precipitated using 50 µl of Saccel (IBL). The radioactivity of the precipitate was determined in a γ -counter.

Determination of [^3H]Inositol Phosphates

After transfection cells were cultivated for 24 h. The DMEM was supplemented with 74 kBq/ml myo-[2- ^3H]-inositol (Amersham Biosciences) and cells were incubated for additional 24 h. Cells were washed with stimulation buffer (culture medium with 10 mM HEPES, 0.5% BSA and 10 mM LiCl) and then stimulated for 60 min at 37°C with bTSH in stimulation buffer. Stimulation medium was removed and cells were lysed with 0.1 M NaOH. By subsequent addition of 0.2 M formic acid and dilution buffer (5 mM sodium tetraborate, 0.5 mM EDTA) the appropriate conditions (pH, ion strength) were adjusted. The lysates were spun and supernatants were subjected to anion exchange chromatography on Sep-Pak Accell™ Plus QMA Cartridges (Waters). [^3H]Inositol 1,4,5-triphosphate was eluted with 0.1 M formic acid and 0.4 M ammonium formate. Radioactivity was determined in a liquid scintillation counter. For normalization, the individual counting results were related to the total amount of myo-[2- ^3H]-inositol incorporated into the HEK293 cells.

Generation of a TSHR serpentine domain model by molecular homology modeling procedures

The available GPCR crystal structures for generation of GPCR homology models in the inactive state are the β 2-adrenergic receptor, rhodopsin, and adenosine-receptor (reviewed in [39–42]). These structures are silenced in their signaling activity by keeping the receptors in a rigid conformation due to different methods: (a) inverse agonists as ligands, (b) modifications by mutations (silencing or thermostabilizing mutations), or (c) fusion with proteins like lysozyme (reviewed in [43]).

Here, we used previously described homology models of the inactive TSHR based on rhodopsin [44] and docking models of TSHR with allosterically bound drug-like antagonist or agonists [33, 45] to estimate potentially participating and signaling sensitive residues of the allosteric binding region. The amino acids for mutagenesis were chosen according to the following criteria: (1) they are in spatial proximity to the known allosteric binding region, (2) they have not been characterized previously by mutagenesis to our knowledge.

To facilitate comparison of different GPCRs, we used both the amino acid numbering scheme of the entire TSHR with its putative signal peptide and the Ballesteros–Weinstein nomenclature [46]. All structure images were produced using PyMOL software (DeLano WL, version 099, San Carlos, CA, USA).

Results

Previously, several mutations of residues covering the allosteric ligand binding pocket in the TSHR (Table 1) were identified, where side-chain variations lead to TSHR activation in the serpentine domain [37]. Our goal in this study was to identify those positions in this region that are sensitive for impairment of TSHR signaling and we mutated amino acids which have not been investigated so far. It is of note, that the TSHR exhibits elevated basal signaling activity, which can be impaired or nullified by in vitro and in vivo mutations. These mutations are termed ‘silencing mutations’ and they are known to decrease also constitutive activation induced by CAMs [44].

We determined basal and TSH-induced cAMP accumulation to characterize signaling properties of mutant TSHRs (Table 2; Fig. 1). Most of the side-chain variations led to cell surface expression levels comparable to wild-type, with the exception of mutation V502A in TMH3 with around 40% of wild-type receptor expression. Each of the ten mutants reached a

maximum level of TSH induced cAMP accumulation of about 80–110% compared to wild-type (set at 100%). Mutation V586I lead to a slight increase in basal activity of 1.7 (± 0.26) and substitution L589V showed a basal activity of 1.29 (± 0.13) compared to wild-type, respectively. Although they are well expressed at the cell surface, eight of the ten tested TSHR variants, V424I (TMH1), L467V (TMH2), V502A (TMH3), L552V (TMH4), Y582A/F (TMH5), Y643A (TMH6), and L665V (TMH7) are characterized by impaired or even nullified basal cAMP values compared to wild-type (Fig. 1). Two of them, L467V (TMH2) and Y643A (TMH6), showed a 5- and 9-fold decreased potency for TSH compared to the wild-type TSHR, respectively (Table 2; Fig. 2). This indicates an influence on TSH-induced cAMP signaling in addition to the effect on basal signaling. Furthermore, these silencing mutants are simultaneously characterized by decreased maximum inositol phosphate second messenger accumulations (between 18 and 70% compared to the wild-type) stimulated by TSH. In conclusion, the here identified sensitive side-chain alterations do selectively affect the maximum of G_q-protein-mediated signaling of TSHR, but not the maximum of signaling capacity mediated by G_s. Six out of the seven positions of silencing mutations are arranged spatially in two different clusters (Fig. 3b, clusters I and II).

Discussion

The allosteric binding region for drug-like small-molecule ligands of the TSHR is comprised of 35 amino acids (Table 1). Evidence for small-molecule binding in this spatial region of the TSHR between the transmembrane helices (TMHs) and the extracellular loop (ECL) 2 was provided in several previous studies [33, 35, 45]. Interestingly, this transmembrane binding pocket region is generally known for family A GPCRs to be sensitive for ligand binding [47]. Our goal in this study was to identify those positions in this region that are sensitive for inactivation of the receptor in order to support the development of low-molecular weight (LMW) antagonists that are able to block pathogenic TSHR activation. Indeed, we here report the identification of mutations characterized by impairment of constitutive signaling activity (silencing) flanking the allosteric binding pocket within two spatial clusters.

Based on the decreased basal activity of the mutants we hypothesize that their wild-type amino acids are important to maintain the basally active wild-type TSHR conformation. Our TSHR homology model predicts that the wild-type amino acids V502 (TMH3), L552 (TMH4), and Y582 (TMH5) are in close proximity to each other. Moreover, amino acid M572 at ECL2 contributes to cluster I of signaling-sensitive amino acids (Fig. 3b). Silencing mutations at this position were previously reported [48]. In particular, the hydrophobic side-chains of V502 and L552 interact with each other between TMH3 and 4, while Y582 from TMH5 interacts with M572 from ECL2 and completes this cluster arrangement. Mutations at these positions indicating that reduction of bulkiness of the side-chains led to silencing of basal signaling. The second significant accumulation of silencing mutations is arranged in a microdomain between TMHs 1, 2, and 7 (cluster II in Fig. 3b). Amino acid mutations at V424 (TMH1), L467 (TMH2), and L665 (TMH7) are characterized by slight variations of bulk of side-chains (Table 2) modifying hydrophobic interactions mandatory for basal signaling activity.

Noteworthy, in contrast to the herein identified silencing mutant Y643A, the substitution Y643F is a CAM [37]. This indicates that dependent on the side-chain substitution, in this case a small alanine versus a bulky aromatic phenylalanine, the TSHR activity can be tuned up or down at position 643. This sensitivity with respect to modification of the bulkiness of the side group also shows that Y643 must be tightly packed between surrounding amino acids in a signaling sensitive region between TMH6, TMH7 and ECL3. Since the Y643F mutant is constitutively active it can be assumed that the hydroxy-group of the tyrosine side

chain is of importance for maintaining the basally active conformation (it likely interacts with the backbone of ECL3) of the TSHR. In contrast, mutations of Y582 at TMH5 to small alanine and bulky phenylalanine revealed no reversed effects, both side-chain variations lead to decreased basal activity (Fig. 1). All these findings highlight Y643 as a potential trigger point for changing a small molecule from an agonist to an antagonist or vice versa by chemical modifications. We conclude from our observations that sensitive positions for inhibition of constitutive TSHR activity are located between TMHs 1, 2, and 7 (cluster II, Fig. 3b), and they are also cumulated at the interfaces between TMHs 3, 4, and 5 close to the small ligand binding site (cluster I, Fig. 3b). This is supported by previous mutagenesis data at TMH3 of the TSHR where mutations that are located between the interfaces of TMH3 to TMH4 and TMH3 to TMH5 caused impaired basal activity [49].

Furthermore, the small-molecule ligand compound 52 (Fig. 4) was previously described to antagonize TSHR activation [33]. It is a neutral antagonist as it inhibits TSH-induced TSHR activity only. Compound 52 shares the same basic thienopyrimidine scaffold with the agonist compound org41841 [35, 36, 50, 51] but differs by the presence of an enlarged substituent on the phenyl-group (Fig. 4). Based on molecular docking studies using a 3D model of the TSHR we predicted that both compounds are localized between the TMHs 3, 4, 5, 6, and ECL2 (Fig. 4). However, the antagonist is located higher in the binding pocket more towards the extracellular region compared to the agonist org41841 (33). Interestingly, the extended substituent of c52 points between TMHs 4, 5, and ECL2 [33] and matches the spatial location where we found the inactivating mutations of residues within cluster I. We conclude that c52 interacts at cluster I and induces a conformation of decreased capability for signaling. Thus both c52 and our mutations in cluster I, are decreasing the signaling capability of the TSHR at the same spatial region. However, the molecular effects of the antagonist and the mutation-induced changes in this region can not completely overlap, because c52 is not characterized as inverse agonist. It should be mentioned that the partial agonist org41841 interacts with residue M637 at TMH6 (Fig. 4) while antagonist c52 can not reach and interact with M637. This is in agreement with the recently reported finding that residue M637 is sensitive for TSHR activation [37] and might explain the agonistic effect of org41841.

In summary, we show that side-chain alterations at seven positions located in close proximity to the potential binding pocket of small-molecule ligands lead to impairment of basal signaling and in particular cases also to a decreased TSH induced activity (both cAMP and IP accumulation) of the TSHR. Interestingly, the majority of identified positions are cumulated in two distinct spatial clusters between TMHs 3, 4, and 5, and TMHs 1, 2, and 7. The here identified amino acids indicate locations where small-molecule antagonists could impair signaling activity. Thus, these results are the base for refined structure–function knowledge that might support rational development of small-molecule inverse agonists or of neutral antagonists for the TSHR in the future. Our new findings are of interest for homologous GPHRs [51] and might have implications for other GPCRs with elevated basal signaling activity [44, 52]. Finally, our study provides interesting information regarding a ligand-binding region that is conserved among the family A GPCRs.

Abbreviations

GPHR	Glycoprotein hormone receptor
LHCGR	Lutropin/choriogonadotropin receptor
FSHR	Follicle stimulating hormone receptor
TSHR	Thyroid stimulating hormone receptor

bTSH	Bovine thyroid stimulating hormone
LH	Luteinizing hormone
CG	Choriogonadotropin
FSH	Follicle stimulating hormone
GPCR	G-protein-coupled receptor
TMH	Transmembrane helix
ECL1/2/3	Extracellular loops 1/2/3
ICLs 1/2/3	Intracellular loops 1/2/3
LRRD	Leucine-rich repeat domain
SD	Serpentine domain
CAM	Constitutively activating mutation
LGRs	Leucine-rich repeat containing GPCRs
7TMRs	Seven-transmembrane spanning receptors
c52	Compound 52
org41841	Organon 41841
DMEM	Dulbecco's modified Eagle's medium
PBS	Phosphate buffered saline
IP	Inositol phosphate

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Fig. 1.

Basal cAMP production of TSHRs harboring mutations in the allosteric ligand binding site. HEK 293 cells were transiently transfected with wt TSHR and mutated TSHRs. cAMP production was measured by RIA as described in Materials and methods. The basal activity of wt TSHR was set at 1 and the basal activity of the mutants is expressed as fold of wt TSHR. Data are given as mean $\pm$ SD of at least two independent experiments, each carried out in duplicate

Fig. 2.

Effect of mutations L467 V and V586I on TSH stimulated cAMP accumulation. TSHR mutants L467V and V586I are shown as examples for mutants with impaired and unaffected TSH stimulation. cAMP concentration as a function of TSH stimulation was determined in HEK 293 cells transiently transfected either with wild-type or mutated TSHR. Measurement was done by RIA as described in the Materials and methods section. Data are given as mean $\pm$ SD. Curves are representative for one out of two independent experiments each done in duplicates

Fig. 3.

Amino acids of the potential allosteric binding pocket for small-molecule ligands in the thyrotropin receptor. The homology model of the transmembrane TSHR serpentine domain visualizes the spatial localization of 35 amino acids (sticks) that participate in the constitution of the transmembrane binding region for small drug-like molecules (TSHR specific numbering starting with the signal-peptide). Colored in *cyan* are residues whose mutations lead to an impaired signaling activity. Amino acids marked with the symbol *asterisk* were investigated in this study. For all other amino acids functional data from mutagenesis studies are published previously. **a** Side view (lateral to the membrane) of the transmembrane homology model. **b** In the top-view (from the extracellular side) only positions of inverse agonistic mutations are labeled and the potential allosteric binding region is highlighted with two translucent *blue fully colored circles*. The accumulation of inverse agonistic mutations (*cyan circles*) occurs between TMHs 3, 4, 5, ECL2 (Cluster I), and between TMHs 1, 2 and 7 (Cluster II)

Fig. 4.

Comparison of poses and spatial location between the small-molecule antagonist c52 and the agonist org41841 within the allosteric binding pocket of TSHR. Both compounds, antagonist c52 (*magenta*) [33] and agonist org41841 (*blue*) [35, 36, 50, 51], are predicted to bind between the helices 3, 4, 5, 6, and ECL2. The chemical structure of c52 differs by the presence of an enlarged substituent on the phenyl-group. This causes c52 to be located spatially higher towards the extracellular region compared to the agonist org41841. The extended substituent of c52 points between helices 4, 5, and ECL2 and matches the spatial location where we found inverse agonistic mutations (*cyan*) of residues within Cluster I. Thus both c52 and our newly identified mutations in cluster I, are decreasing the signaling capability of the TSHR at the same spatial region. However, the molecular effects of the antagonist and the mutation induced changes can not completely overlap, because c52 is not an inverse agonist. It should be mentioned that the agonist org41841 is interacting with residue M637 at TMH6 (*red stick*) while c52 can not reach this location. This is in agreement with the recently reported finding that residue M637 is an important sensitive trigger point for TSHR activation [37] and might explain the agonistic effect of org41841

Table 1

Summary of amino acids constituting the transmembrane binding pocket for small-molecule ligands of the TSHR

Location	Ballesteros and Weinstein numbering	Amino acid hTSHR	Contacts to org41841 (agonistic)	Contacts to c52 (antagonistic)
TMH1	1.35	L417		
	1.39	V421		
	1.42	V424*		
TMH2	2.53	M463		
	2.56	Y466		
	2.57	L467*		
	2.60	I470		
	2.64	D474		
TMH3	3.32	T501		
	3.33	V502*	+	+
	3.36	S505	+	–
	3.37	E506		
TMH4	3.40	V509		
	4.56	L552*	–	+
ECL2		I568	–	+
		L570		
		P571		
		M572	–	+
TMH5	5.39	Y582*	–	+
	5.40	I583		
	5.43	V586*	+	+
	5.44	L587		
TMH6	5.46	L589*		
	5.47	N590		
	6.48	M637	+	–
	6.50	P639		
	6.51	I640	+	+
	6.52	S641		
	6.54	Y643*		
TMH7	6.56	L645		
	6.55	A644		
	6.59	I648		
	7.39	V664		
	7.40	L665*		
	7.42	Y667	+	+

This overview is generated from observations at the TSHR homology model (Fig. 3) and by previous studies [33, 36, 45] concerning potential participation of amino acids that cover the allosteric small ligand binding pocket in the transmembrane region. The numbers of positions are provided according to the Ballesteros and Weinstein numbering system for GPCRs [46] and in parallel the TSHR specific residue and number

starting with the first amino acid of the signal-peptide. Amino acids investigated in this study are bold and marked with asterisk, for all other amino acids experimental data were published previously (see GPHR information system: <http://www.ssfa-gphr.de> [53, 54]). We also assigned contacts (+) between the TSHR and the small ligands bound to the allosteric binding site as shown in Fig. 4

Functional characterization of TSHR mutations

Table 2

Location	Transfected construct	Ballesteros and Weinstein numbering	Cell surface expression	cAMP accumulation			IP accumulation	
				FACS% of TSHR wt	EC50 nM	TSH stimulated % of TSHR	TSH stimulated % of TSHR	
	wt TSHR			100	1.2 (0.8–1.7)	100	100	
TMH1	V424I	1.43		102 ± 10	3.0 (2.2–4.1)	98 ± 8	53 ± 4	
TMH2	L467V	2.57		85 ± 8	11.2 (10.4–12.1)	105 ± 14	19 ± 1	
TMH3	V502A	3.33		44 ± 4	4.3 (3.9–4.7)	84 ± 10	13 ± 0	
TMH4	L552V	4.56		90 ± 5	1.0 (0.8–1.4)	84 ± 19	79 ± 11	
TMH5	Y582A	5.39		71 ± 5	3.7 (2.6–5.2)	106 ± 19	45 ± 17	
TMH5	Y582F	5.39		86 ± 8	1.9 (1.3–2.8)	108 ± 12	114 ± 18	
TMH5	V586I	5.43		107 ± 6	1.1 (0.7–1.7)	82 ± 7	120 ± 6	
TMH5	L589V	5.46		100 ± 12	1.1 (1.0–1.2)	90 ± 9	110 ± 11	
TMH6	Y643A	6.54		99 ± 9	6.0 (4.5–8.0)	93 ± 9	47 ± 4	
TMH7	L665V	7.40		95 ± 11	2.0 (2.0–2.0)	100 ± 10	71 ± 6	

HEK 293 cells were transfected with wt TSHR and mutated TSHRs. The TSHR cell surface expression level was quantified on a FACS flow cytometer. Receptor expression was determined by the mean fluorescence intensity by comparison to wt TSHR. Stimulation was carried out with increasing concentrations of TSH. Results for cAMP accumulation were measured by RIA. Quantification of IP accumulation was done by [³H]inositol incorporation studies. Data are given as mean ± SD of at least two independent experiments, each carried out in duplicate. EC50 values are shown as geometric mean (95% confidence limits). The pcDNA3 vector was used as a control