

# TSH receptor mutation V509A causes familial hyperthyroidism by release of interhelical constraints between transmembrane helices TMH3 and TMH5

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## Abstract

Mutations of the human thyrotrophin receptor (TSH-R) are a cause of thyroid adenomas and hyperthyroidism. Here we study mechanisms of receptor activation in a genomic TSH-R variant V509A located in transmembrane helix (TMH) 3, which we identify in a family with congenital hyperthyroidism, multiple adenomas and follicular thyroid cancer. Using molecular modelling and dynamic simulation, we predicted the release of amino acid residue A593 (located opposite in domain TMH5) from a tight 'knob-and-hole' interaction with TMH3, physiologically constrained in the native receptor state by the bulky side chain of V509. To experimentally validate this concept, we generated mutant TSH-R expression constructs for functional *in vitro* studies. TSH-R mutant

V509A showed a 2·8-fold increase in basal cAMP production, confirming constitutive TSH-R activation. The addition of a second site suppressor mutant A593V to TSH-R V509A resulted in the normalization of basal cAMP release, and the dose-responsiveness to TSH ligand was maintained. These data thus demonstrate that TSH-R V509A activation is caused by the release of TMH3–TMH5 interhelical constraints, while the native TSH-R conformation is re-stabilized by the introduction of a spacious valine residue at position 593. In conclusion, we delineate a novel mechanism of constitutive TSH-R activation, leading to thyroid hyperfunction and neoplasia.

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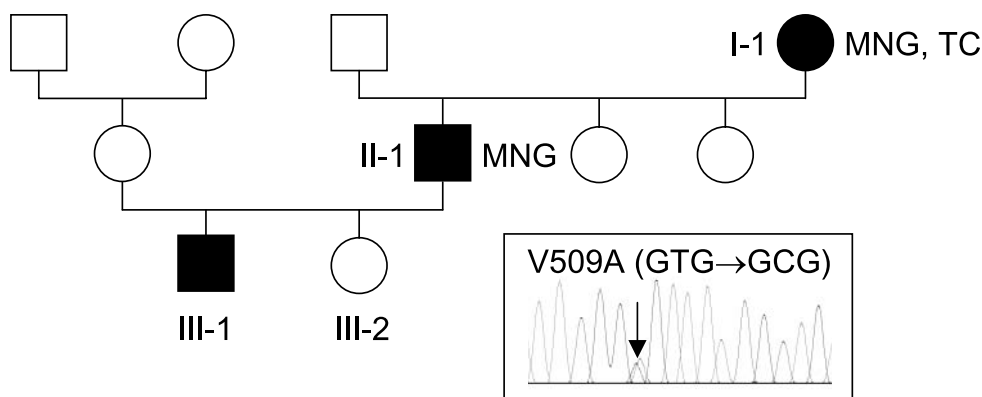
## Introduction

The thyrotrophin receptor (TSH-R) belongs to the family of G protein-coupled receptors (GPCRs) containing seven transmembrane helices (TMH). Since 1994, activating germline mutations of the TSH-R have been identified as a cause of familial non-autoimmune thyrotoxicosis (Duprez *et al.* 1994). Persistent hyperthyroidism in affected individuals is often resistant to conventional treatment, eventually requiring complete thyroidectomy or ablative radiotherapy (Corvilain *et al.* 2001). Similarly, activating somatic mutations of the TSH-R have been found in toxic thyroid adenomas. There is evidence that constitutively active TSH-R variants are involved in thyroid cell growth and tumour development (Parma *et al.* 1993, Russo *et al.* 1995b, 1997, Spambalg *et al.* 1996, Fournes *et al.* 1998, Ludgate *et al.* 1999).

Activation of GPCRs is a dynamic process involving the release of inactive state constraints and sequential

agonist-induced conformational changes until the active receptor state is reached (Schoneberg *et al.* 1999, Gether 2000, Schulz *et al.* 2000). Ligand-induced TSH-R activation mainly results in stimulation of adenylate cyclase with an increase in intracellular cyclic AMP (cAMP). Similarly, constitutively active TSH-R variants exhibit increased basal adenylate cyclase activity compared with the wild-type TSH-R (Corvilain *et al.* 2001). Natural activating TSH-R variants are thought to mimic the active conformation of the wild-type TSH-R and may spontaneously adopt a conformation able to activate G protein-dependent signalling pathways (Parnot *et al.* 2002). However, the precise molecular mechanisms of TSH-R activation are not completely understood.

We here identify a German family with congenital hyperthyroidism, multiple adenomas and follicular thyroid cancer, associated with TSH-R genomic variant V509A. In this first described TSH-R germ line mutation (Duprez *et al.* 1994), we use molecular modelling and dynamic



**Figure 1** Family with non-autoimmune hyperthyroidism due to activating TSH-R mutation V509A. Closed symbols indicate patients with hyperthyroidism and heterozygous TSH-R mutation. Open symbols indicate healthy subjects. MNG, multinodular goitre; TC, follicular thyroid carcinoma. Insert: genomic TSH-R mutation V509A, located in exon 10.

simulation to predict the structure and function of the variant TSH-R protein. Site-directed mutagenesis and functional *in vitro* studies further indicate that the release of residue A593 in TMH5 from the steric 'knob-and-hole' interaction with TMH3 is a pivotal element for constitutive TSH-R activation observed in this kindred.

## Subjects and Methods

### *Patient characteristics and laboratory evaluation*

The index patient (subject III-1, Fig. 1) is a boy of Caucasian origin who at the age of four years presented with hyperactivity and insomnia starting at the age of 6 months. His height was 121.8 cm (97th percentile), his weight was 22.5 kg (90–97th percentile), his heart rate was 90/min and his blood pressure was 129/62 mmHg. Elevated thyroxine (182.75 nmol/l, normal range 61.4–117.2 nmol/l), triiodothyronine (3.6 nmol/l, normal range 1.83–3.30 nmol/l) and low thyrotrophin (TSH) (0.03 mU/l, normal range 0.5–5.5 mU/l) were detected, using commercial immunometric reagent kits (Vitros, Ortho-Clinical Diagnostics, Amersham, Bucks, UK). TSH receptor antibodies (TRAKhuman, Brahms Diagnostica, Henningstedt, Germany), thyroglobulin antibodies (VeliSa, Vita Diagnostica, Freiburg, Germany), and thyroid peroxidase antibodies (VeliSa, Vita Diagnostica) were negative. Thyroid volume, as determined by ultrasound, was increased to 4.4 ml (>99th percentile compared with age- and gender-specific controls (Liesenkotter *et al.* 1997)), and the bone age was accelerated to 5.5 years.

The patient's father (subject II-1, Fig. 1) had suffered from clinical signs of hyperthyroidism since childhood, but diagnosis and antithyroid treatment was not established until 18 years of age. He underwent thyroidectomy for multinodular goitre at the age of 36 years. The mother and sister (subject III-2) of the index patient were healthy. His

paternal grandmother (subject I-1) had undergone thyroidectomy because of hyperthyroidism and multinodular goitre at the age of 60 years. After the diagnosis of follicular thyroid cancer had been established by histopathology, she received ablative radioiodine therapy, and her further clinical course was uneventful. Informed consent was obtained for all analyses from patients or their parents, and studies were performed in accordance with the Declaration of Helsinki.

### *TSH-R sequence analysis*

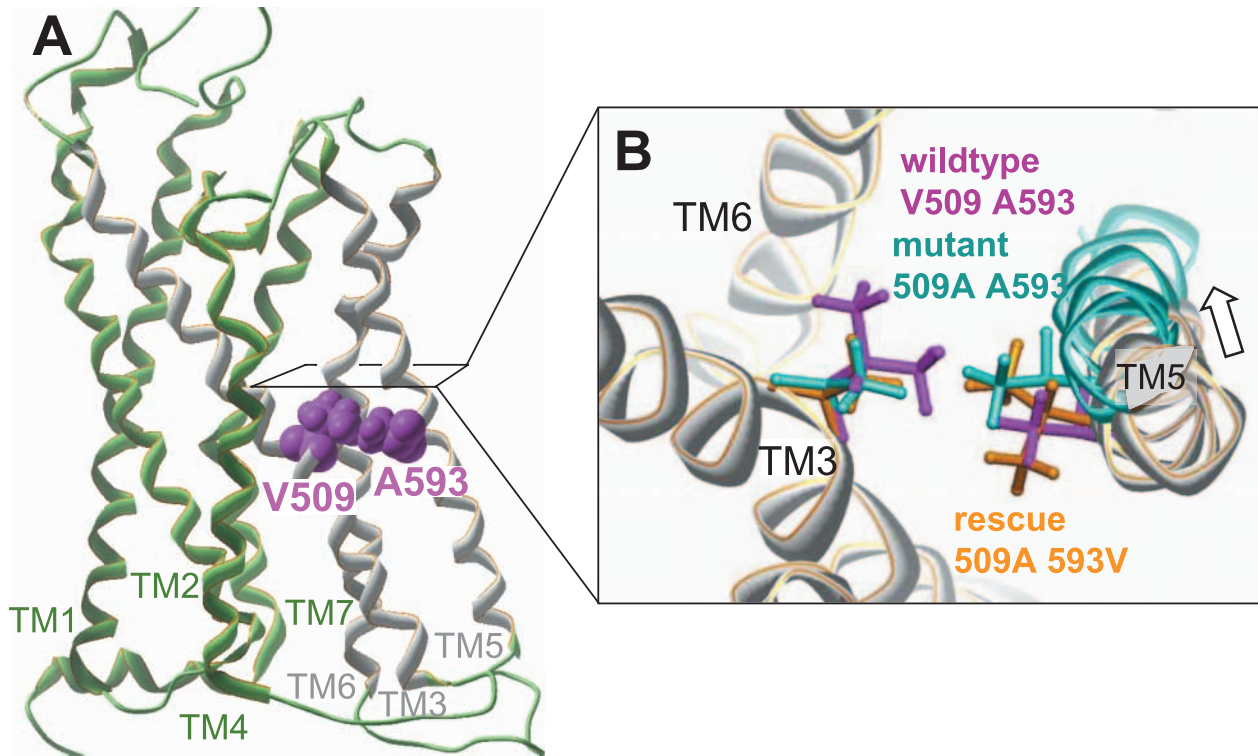
Sequence analysis of the human TSH-R gene (GenBank accession M31774) in patients was performed as described (Duprez *et al.* 1994) using genomic DNA from peripheral blood lymphocytes (DNeasy kit, Qiagen, Hilden, Germany) as template. Identical primers were used for genomic polymerase chain reaction and automated DNA sequencing (ABI Prism 7700, Applied Biosystems, Foster City, CA, USA).

### *TSH-R expression constructs*

Wild-type TSH-R complementary DNA containing the entire coding sequence (2295 bp, kind gift of Dr Loos, Ulm, Germany) was cloned into the BamHI and EcoRI sites of pIRES (Clontech, Palo Alto, CA, USA). TSH-R plasmid constructs containing mutations V509A and A593V were generated by site-specific PCR mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA), using wild-type TSH-R and TSH-R V509A plasmids as amplification templates respectively. All constructs were confirmed by bi-directional sequencing.

### *Cell culture and in vitro transfection*

Signal transduction studies were performed in human embryonic kidney cells (HEK) 293 cells, grown in



**Figure 2** (A) Three-dimensional model of TSH-R transmembrane (TM) helices. Space-filled representation of side chains demonstrates the tight intertwined interactions between V509 (TMH3) and A593 (TMH5). (B) Detailed extracellular view of TMH3 (TM3) and TMH5 (TM5). Superposition of conformation frames from molecular dynamics for wild-type TSH-R (magenta), mutant V509A (cyan) and second-site mutant V509A/A593V (orange). The constitutive active mutant (cyan) allows closer distance between positions 509 (TMH3) and 593 (TMH5), with subsequent conformational change of TMH5 (arrow). The functional 'rescue' mutant (orange) reconstitutes a distance similar to wild-type TSH-R.

DMEM/10% FCS/1% penicillin-streptomycin. Cells ( $1 \times 10^5$ /well) were seeded in 12-well dishes previously coated with fibronectin (50 ng/ml; Sigma, St Louis, MO, USA). Transient transfection with TSH-R constructs (1  $\mu$ g plasmid/well) was performed 24 h after plating (FUGENE6, Roche, Mannheim, Germany). Transfections were performed in triplicate, and each independent experiment was performed at least twice. TSH-R expression in cell lysates was studied by Western blotting using mouse monoclonal anti-TSH-R antibody (3H10, Brahms Diagnostica, Berlin, Germany) and the ECL system (Amersham, Freiburg, Germany). In addition, TSH-R cell surface expression was analysed by immunofluorescence microscopy (Olympus IX71) in transiently transfected HEK 293 cells using a monoclonal antibody directed against the extracellular part of the TSH-R (T5-34 antibody, 1:2000) and a FITC-conjugated secondary antibody (Alexa488, Molecular Probe, Eugene, CA, USA) as described (de Roux *et al.* 1996b).

#### cAMP assay

The accumulation of cyclic adenosine monophosphates (cAMP) in HEK 293 cells transfected with different

TSH-R constructs was measured to determine basal and stimulated receptor activity. Twenty-four hours after transfection, culture medium was replaced for 60 min by DMEM containing 0.4 mM 3-isobutylmethylxanthine (Sigma). Subsequently, cells were lysed with 1 M perchloric acid at 4 °C, and cAMP accumulation in the culture medium was measured by radioimmunoassay (cAMP [ $^{125}$ I] Biotrak assay system, Amersham, Germany). In a second set of experiments, dose-response relations of cAMP production were established by incubation of transfected cells in the presence of 0.1, 1, and 10 mU/ml bovine TSH (Sigma). cAMP production (pmol/well) is shown as the mean  $\pm$  S.D. of triplicate measurements, and all experiments were performed at least twice, with identical results.

#### Molecular modelling of TSH-R structure

The construction of the TSH-R model has been described previously (Neumann *et al.* 2001). The starting structure of the transmembrane helices of the human TSH-R was based on the crystal structure of bovine rhodopsin as template (Palczewski *et al.* 2000). The TSH-R structure model was computed with particular emphasis on the

transmembrane helices, without the large amino-terminal domain but including extra- and intracellular loops. All model components were assembled with the biopolymer module of the Sybyl program package (TRIPOS Inc., St Louis, MO, USA). Molecular dynamic simulations were performed at 300 K for 1 ns using the AMBER 6.0 force field (Case *et al.* 1998), where only hydrogen bonds of the TMH backbones maintaining the TMH helices were restrained.

## Results

### *Familial hyperthyroidism and thyroid neoplasia*

In the index patient (Fig. 1, individual III-1), mild hyperthyroidism was diagnosed at the age of four years, when antithyroid treatment was initiated. During his subsequent clinical course, carbimazole doses were adjusted to maintain thyroxine in the upper normal range, without signs or symptoms of hyperthyroidism. Medical treatment was well tolerated. At the age of 11 years and 6 months, ultrasound revealed an increased thyroid volume (8.5 ml, corresponding to >97th percentile of age- and gender-specific controls (Liesenkotter *et al.* 1997)) without evidence of thyroid nodules. During follow-up, his longitudinal height and weight were above the 97th percentile (Prader *et al.* 1989) with a height of 178 cm, weight of 84.4 kg, and body mass index of 27.2 kg/m<sup>2</sup> at the age of 12 years. His bone age was accelerated to 16 years. The predicted adult height is 181.6 cm compared with midparental target height of 176.8 cm.

His father (Fig. 1, individual II-1) had undergone complete thyroidectomy at the age of 36 years because of recurrent hyperthyroidism and multinodular goitre. Histopathological evaluation showed multiple microfollicular adenomas in both thyroid lobes. At the age of 47 years, he currently receives 100 µg L-thyroxine per day, and his thyroid function is normal. His mother had no history of thyroid disease and her thyroid hormone levels were within the normal range. The paternal grandmother (Fig. 1, individual I-1) was diagnosed with nodular goitre and hyperthyroidism in adulthood. Subtotal thyroidectomy was performed in the early 1980s when the patient was 60 years old. Histopathological evaluation revealed a follicular oncocytic thyroid carcinoma, sized 20 × 15 mm, in the left lobe, with capsular infiltration and angioinvasion (pT3N0M0; International Union Against Cancer, Geneva, Switzerland, 2002), as well as multiple microfollicular adenomas in both lobes. Subsequently, she received ablative radioiodine therapy, and her clinical follow-up was unremarkable.

### *TSH-R germline mutation V509A*

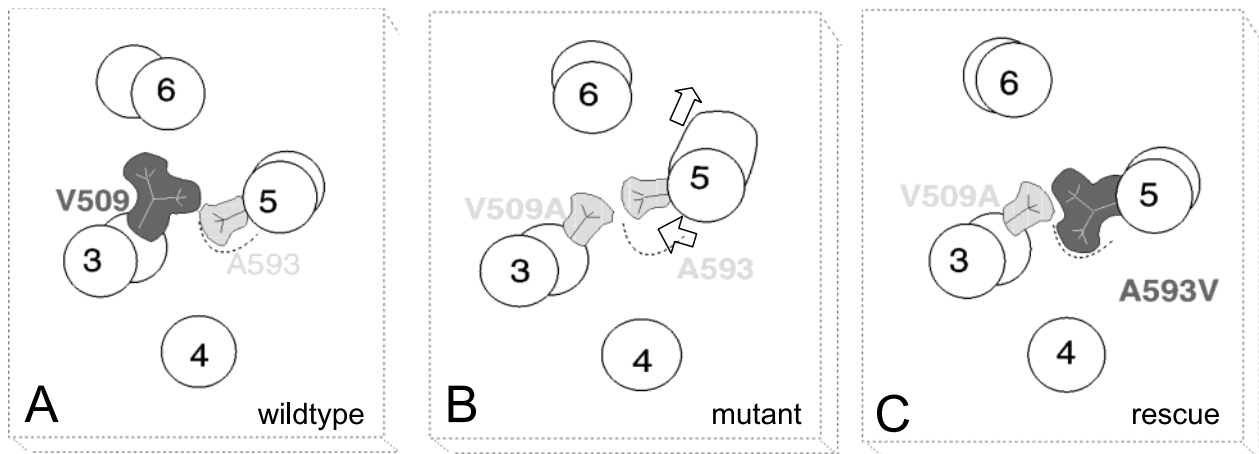
The absence of thyroid autoantibodies, early clinical onset, and familial clustering suggested hereditary hyperthy-

roidism in the index patient. We performed a genetic analysis of the TSH-R, identifying heterozygous genomic TSH-R mutation V509A in the index patient and affected family members (Fig. 1, insert). Transition of T (GTG) to C (GCG) in codon 509 (exon 10) results in replacement of valine by alanine (V509A) in TMH3 of the TSH-R. V509A was the first human genomic TSH-R mutation described (Duprez *et al.* 1994). The wild-type TSH-R sequence was found in the mother of the index patient.

### *Molecular modelling of TSH-R V509A receptor structure and function*

To account for the activation of the V509A TSH-R at the structural level, molecular dynamic (MD) simulation and modelling studies were performed. For the wild-type TSH-R a tight 'knob-and-hole' interaction between TMH3 and TMH5 was identified for this particular receptor area with residue A593 as the counterpart of position 509 (Fig. 2). During MD simulations, the distance between the C-alpha atoms of 509A and A593 (approximately 1 Ångstrom) was considerably shorter than in the wild-type TSH-R. In detail, after equilibration the distances between the C-alpha atoms of position 509 and 593 were stabilized during the trajectories for the V509A mutant in a range between 5.4 and 6.4 Ångstroms and for the wild-type TSH-R in the range of 6.5–7.7 Ångstroms. In the wild-type TSH-R the branched side chain of Val509 ('knob') in TMH3 constrains residue Ala593 (hydrophobic 'hole') in the opposite position in TMH5 (Fig. 3A). The Ala593 side chain acts as an intertwined complementary counterpart participating at a hydrophobic hole that is coated by the other hydrophobic residues Phe594, Leu539 at TMH5 and Tyr510, Thr513 at TMH3. Mutation Ala509, deleting the bulky side chain, releases the intertwining and permits the escape of Ala593 from the hydrophobic hole. Thus, the intracellular movement of TMH5 and intracellular loop 3 may lead to activation of the mutant TSH-R (Fig. 3B).

We next developed and tested an experimental double mutant V509A/A593V TSH-R model in which the 'knob' functionality is restored by valine at position 593, with the branched side chain fixing A509 and a methyl group filling in its hydrophobic hole (Fig. 3C). MD simulation showed a similar distance between TMH3 and TMH5 for wild-type and double mutant TSH-R. In effect, the introduction of a second site mutant, A593V, is predicted to restabilize the TSH-R in its native conformation. Taken together, the modelling results suggest that in TSH-R V509A a release of tight interactions between amino acid residues V509 and A593 results in an increased mobility of TMH3 and TMH5, causing constitutive TSH-R activation.

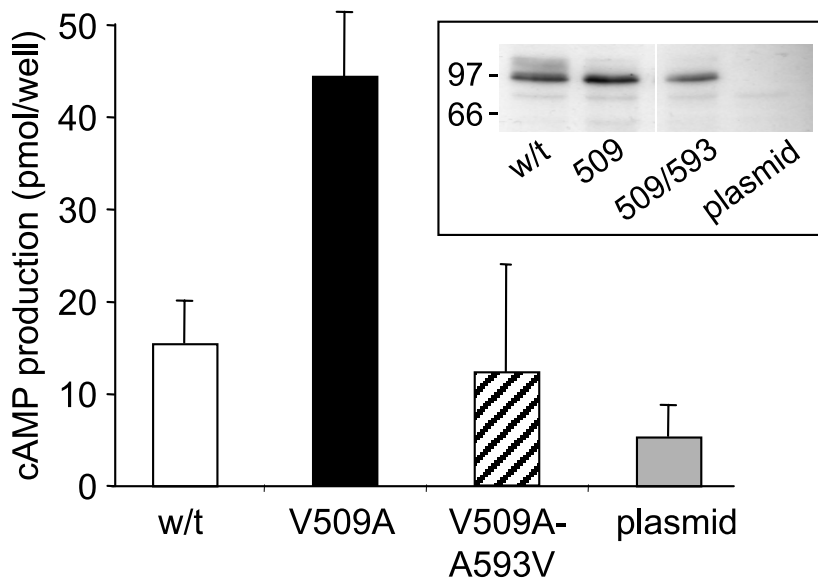


**Figure 3** Functional model of interaction between TSH-R transmembrane domains TMH3 and TMH5. (A) In wild-type TSH-R, V509 ('knob') fixes the oppositely located Ala593 (TMH5) in a hydrophobic hole. (B) The small alanine side chain in mutant TSH-R V509A permits escape of Ala593, thus allowing intracellular movement of TMH5. (C) In TSH-R V509A/Ala593V, functionality of the knob is taken over by valine at position 593 as the branched side chain of Val593 fixes alanine at TMH3, and a methyl portion of V593 fills in the hydrophobic hole. In effect, the designed second site mutant A593V stabilizes the native TSH-R conformation.

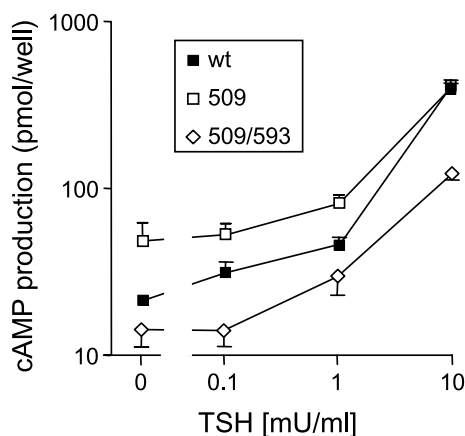
#### *In vitro TSH-R V509A activation and signal transduction*

The effects of mutation V509A on basal and ligand-induced TSH-R receptor activation was studied *in vitro* in human embryonic HEK 293 cells expressing wild-type or mutated TSH-R (Fig. 4, insert). A significant (2.8-fold) increase in basal intracellular

cAMP accumulation was observed in the mutated TSH-R V509A compared with the wild-type TSH-R (Fig. 4) in the absence of TSH, indicating constitutive TSH-R activation. Incubation with different concentrations of TSH showed a similar increase in cAMP production in cells transfected with wild-type TSH-R and the TSH-R V509A, demonstrating dose-



**Figure 4** Basal cAMP production in HEK cells transfected with TSH-R constructs, showing 2.8-fold cAMP levels for TSH-R V509A as compared with wild-type (w/t) TSH-R. Note the reduction of cAMP production to base line levels in double mutant V509A/A593V TSH-R. Insert: expression of recombinant TSH-R in HEK 293 cells, as detected by immunoblot. Plasmid denotes control transfection with empty plasmid vector. 97 and 66 denote 97 and 66 kDa.



**Figure 5** Dose-dependent *in vitro* cAMP production in the presence of TSH ligand in HEK 293 cells expressing wild-type (wt) or mutant TSH-R.

dependent ligand-induced proximal signal transduction (Fig. 5).

#### Functional 'rescue' of V509A mutant TSH-R by A593V

To experimentally validate the proposed concept of TSH-R V509A activation, a second site TSH-R mutation was introduced into TMH5, replacing alanine in position 593 by valine, thus generating the reciprocal double mutant V509A/A593V. Signal transduction of this double mutant TSH-R was analysed in transfected HEK 293 cells and compared with the wild-type TSH-R and the V509A TSH-R variant. V509A/A593V TSH-R was characterized by a lower basal cAMP production than V509A TSH-R, comparable with the wild-type TSH-R activity (Fig. 4) and A593V TSH-R activity (not shown). Dose-responsiveness to exogenous TSH was maintained in the double mutant V509A/A593V TSH-R (Fig. 5). Similar cell surface expression of wild-type, mutant and double mutant TSH-R was confirmed by immunofluorescence microscopy in transiently transfected HEK 293 cells, using a monoclonal antibody directed against the TSH-R extracellular domain (Fig. 6) which cannot rule out subtle

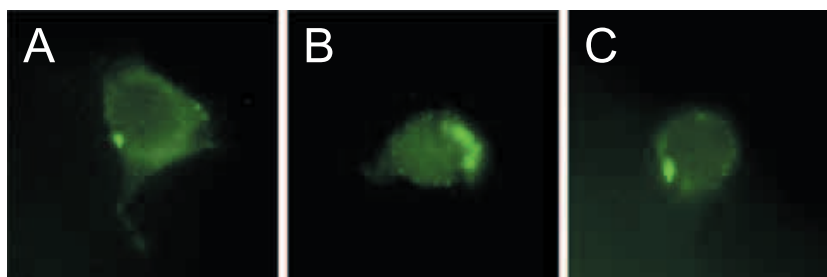
quantitative differences of receptor expression. Taken together, the introduction of valine in position 593 (TMH5) reverses the constitutive activation of mutant V509A TSH-R to normal wild-type TSH-R levels.

#### Discussion

Congenital hyperthyroidism in this family is caused by a germline mutation of the TSH receptor, V509A. This mutation was the first activating genomic TSH-R variant ever described (Thomas *et al.* 1982, Duprez *et al.* 1994). In comparison with the initial French family, thyrotoxicosis in our index patient developed much earlier and represents the youngest symptomatic patient carrying the V509A TSH-R variant reported so far.

Patients with activating genomic TSH-R mutations are characterized by a variable clinical expression of hyperthyroidism (Corvilain *et al.* 2001). Disease activity in affected individuals correlates with the degree of constitutive receptor activation, as determined by basal cAMP production, thus establishing a phenotype-genotype relationship. Severe hyperthyroidism has been found in genomic TSH-R variants with excessive basal cAMP production, presenting a fourfold to sevenfold increase compared with the wild-type TSH-R (Kopp *et al.* 1995, de Roux *et al.* 1996a, Fuhrer *et al.* 1997, Holzapfel *et al.* 1997, Tonacchera *et al.* 2000). In contrast, in our kindred basal cAMP accumulation in the V509A TSH-R was only increased by 2.8-fold, similar to earlier studies using different *in vitro* cell systems (Duprez *et al.* 1994, Kosugi *et al.* 2000, Fuhrer *et al.* 2003). Likewise, thyroid nodules and goitre develop earlier in patients carrying TSH-R variants with high constitutive receptor activation (Kopp *et al.* 1995, de Roux *et al.* 1996a, Fuhrer *et al.* 1997, Holzapfel *et al.* 1997, Tonacchera *et al.* 2000) compared with V509A TSH-R, as described in this and previous reports (Thomas *et al.* 1982, Leclere *et al.* 1997).

Structure-function analysis of TSH-R substitutions has provided insight into the molecular mechanisms of TSH-R activation (Biebermann *et al.* 1998, Govaerts *et al.* 2001, Neumann *et al.* 2001, Kleinau *et al.* 2004, Urizar



**Figure 6** Cell surface expression of (A) wild-type TSH-R, (B) TSH-R V509A/A593V, and (C) TSH-R V509A, as detected by immunofluorescence after *in vitro* transfection of HEK 293 cells, using a monoclonal antibody directed against the TSH-R extracellular domain.

*et al.* 2005). Three activating TSH-R mutations located at TMH3 have been identified to date: V509A, S505R/N, and L512R. In our molecular TSH-R model, all these TMH3 residues point into the receptor core region located between the transmembrane helices. For positions 505 and 512 it is probable that bulkier or more hydrophilic substitutions are repulsing TMH6/TMH7 from TMH3, thereby changing the TSH-R from the constrained native state to the activated state. Using a double mutant strategy it was shown that no hydrogen bond interaction exists between S508 in TMH3 and R633 in TMH3 (Neumann *et al.* 2001). Repulsing scenarios by the introduction of larger amino acids have been described for TMH5 and TMH6 residues of the TSH-R (Biebermann *et al.* 1998). However, this scenario seems unlikely for V509A observed in our family, as the alanine substitution exhibits a reduced size and (weak) hydrophobic properties.

Other mechanisms seem to be involved in the interaction between TMH3 and TMH5 in the centre of the transmembrane region. Molecular modelling of natural GPCR variants, including the gonadotrophin-releasing hormone receptor (Karges *et al.* 2003), has improved the understanding of the GPCR function in human disease. We therefore performed a computed modelling of the TSH-R variant V509A, suggesting that replacement of valine 509 by alanine causes a loss of the tight 'knob-and-hole' interaction between V509 (TMH3) and A593 (TMH5), thereby releasing interhelical constraints to increase the mobility of TMH3 and TMH5. As signal transduction is mainly transmitted by residues in the third intracellular loop (Kosugi *et al.* 1994, Biebermann *et al.* 1998, Vlaeminck-Guillem *et al.* 2002), the intracellular movement of TMH5 may thus initiate stimulation of adenylate cyclase activity.

The experimental introduction of second site suppressor mutations in conjunction with molecular modelling has been used to identify intramolecular GPCR interactions in the TSH-R (Neumann *et al.* 2001) and vasopressin-V2 receptor (Schulein *et al.* 2001, Oksche *et al.* 2002). We generated a double mutant, V509A/A593V TSH-R, for functional rescue of V509A. The 'fixed-and-released' interaction hypothesis suggested by our modelling data was experimentally addressed by replacing alanine 593 in TMH5 by valine, leading to normalization of basal cAMP production and TSH-R activity. Our results demonstrate that specific intertwined 'knob-and-hole' patterns are stabilizing the interaction between residue V509 (TMH3) and A593 (TMH5) to maintain the TSH receptor in its native conformation, and that release of the intertwining in this particular region turns the TSH-R into an active conformation. As residues 509 and 593 are highly conserved in all glycoprotein hormone receptors (GPHRs), this activation mechanism may apply to all GPHRs.

Patients with activating genomic TSH-R mutations and persistent hyperthyroidism may have a predisposition for thyroid neoplasia, since the constitutive activation of signal

transduction pathways stimulates cell proliferation and transformation (Russo *et al.* 1995a, Fournes *et al.* 1998, Ludgate *et al.* 1999). Constitutive activity of the TSH-R mainly increases stimulation of adenylate cyclase but rarely also affects the phospholipase C pathway (Parma *et al.* 1995). Activating somatic mutations of the TSH-R have been found in thyroid neoplasia (Spambalg *et al.* 1996, Russo *et al.* 1997, 1999, Mircescu *et al.* 2000) but until now thyroid carcinoma has not been reported in individuals carrying a genomic activating TSH-R mutation. The low prevalence for thyroid cancer in affected individuals with activating germline TSH-R mutations may be partially explained by early thyroidectomy due to thyrotoxicosis (Thomas *et al.* 1982, Leclere *et al.* 1997). In addition, malignant transformation may require additional (i.e. somatic) mutations, but genetic events involved in this process have not been identified (Russo *et al.* 1995a, 1997, 1999). In contrast, the occurrence of nodular transformation is a common finding in patients with activating germline TSH-R variants (Kopp *et al.* 1995, Tonacchera *et al.* 1996, Leclere *et al.* 1997). In the general population, the risk for thyroid cancer is increased in patients with multinodular goitre (From *et al.* 2000). From a clinical point of view, medical and imaging follow-up to detect thyroid neoplasia seems prudent in individuals with activating TSH-R mutations who do not undergo ablative therapy.

In summary, we propose and experimentally validate a novel mechanism of constitutive GPCR activation in TSH-R variant V509A, leading to hyperthyroidism and a predisposition to thyroid neoplasia in affected patients.

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