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BRIEF REPORT

A Familial Thyrotropin (TSH) Receptor Mutation Provides *in Vivo* Evidence that the Inositol Phosphates/Ca^{2+} Cascade Mediates TSH Action on Thyroid Hormone Synthesis

Helmut Grasberger, Jacqueline Van Sande, Ahmad Hag-Dahood Mahameed, Yardena Tenenbaum-Rakover, and Samuel Refetoff

Departments of Medicine (H.G., S.R.) and Pediatrics (S.R.) and Committee on Genetics (S.R.), The University of Chicago, Chicago, Illinois 60637; Institute of Interdisciplinary Research (J.V.S.), University of Brussels, 1070 Brussels, Belgium; Clalit Health Service (A.H.-D.M.), Um-El Fahem 30010, Israel; Pediatric Endocrine Unit (Y.T.-R.), Ha'Emek Medical Center, Afula 18101, Israel; and Technicon Faculty of Medicine (Y.T.-R.), Haifa 31096, Israel

Context: In the human thyroid gland, TSH activates both the cAMP and inositol phosphates (IP) signaling cascades via binding to the TSH receptor (TSHR). Biallelic TSHR loss-of-function mutations cause resistance to TSH, clinically characterized by hyperthyrotropinemia, and normal or reduced thyroid gland volume, thyroid hormone output, and iodine uptake.

Objective: We report and study a novel familial TSHR mutation (L653V).

Results: Homozygous individuals expressing L653V had euthyroid hyperthyrotropinemia. Paradoxically, patients had significantly higher 2-h radioiodide uptake and 2- to 24-h radioiodide uptake ratios compared with heterozygous, unaffected family members, suggesting an imbalance between iodide trapping and organification. In transfected COS-7 cells, the mutant TSHR had normal surface expression, basal activity, and TSH-binding affinity, equally (2.2-fold) increased EC_{50} values for TSH-induced cAMP and IP accumulation, and normal maximum cAMP generation. In contrast, the efficacy of TSH for generating IP was more than 7-fold lower with the mutant compared with wild-type TSHR.

Conclusions: We identified and characterized a TSHR defect, preferentially affecting the IP pathway, with a phenotype distinct from previously reported loss-of-function mutations. Results provide the first *in vivo* evidence for the physiological role of the TSHR/IP/Ca^{2+} cascade in regulating iodination. According to systematic *in vitro* mutagenesis studies, other TSHR mutations can result in even complete loss of IP signaling with retained cAMP induction. We hypothesize that such TSHR mutations could be the cause in unexplained partial organification defects. **(*J Clin Endocrinol Metab* 92: 2816–2820, 2007)**

THROUGH INTERACTION with TSH, the receptor (TSHR) plays a pivotal role in regulating thyroid gland physiology. This heptahelical receptor with a large extracellular ligand-binding domain couples to G_s and G_q (1) and activates both G_s/cAMP and G_q/phospholipase C/inositol phosphates (IP)/Ca^{2+} cascades in human thyroid cells and slices (2, 3). The cAMP pathway mediates TSH stimulation of 1) hormone secretion by increasing macropinocytosis and micropinocytosis of thyroglobulin, 2) growth and differentiation of follicular cells, and 3) iodide uptake via transcription of the sodium iodide symporter. The IP/Ca^{2+} pathway regulates hormone synthesis by stimulating H_2O_2 generation, required for iodide organification and oxidative coupling of iodotyrosines into iodothyronines. It also rapidly activates apical iodide efflux from thyrocytes into the follicular lumen (for review, see Refs. 4 and 5).

Biallelic, partial TSHR loss-of-function mutations have been identified in patients with resistance to TSH (RTSH) characterized by congenital hyperthyrotropinemia, normal or low free thyroid hormone (TH) levels, and normal-sized or hypoplastic thyroid gland with low thyroidal radioactive iodine uptake (RAIU) (for review, see Ref. 6). These hallmarks of RTSH were attributed to diminished cAMP signaling capacity. However, the dual activation by TSH of the cAMP and IP/Ca^{2+} pathways implies that specific loss-of-function TSHR mutations could lead to differential effects and ultimately distinct clinical subtypes of RTSH.

We report a novel TSHR mutation with imbalanced impairment of the cAMP and IP/Ca^{2+} pathways that could be demonstrated *in vitro* and *in vivo*.

Subjects and Methods

Case reports

The proposita (subject 4) (Fig. 1A), of Arab-Muslim descent, was born to first cousins. Thyroid function tests (TFTs), performed to evaluate sinus tachycardia, revealed markedly elevated serum TSH (53 mU/liter)

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Abbreviations: bTSH, Bovine TSH; ECL3, third extracellular loop; IP, inositol phosphates; RAIU, radioactive iodine uptake; RTSH, resistance to TSH; TFT, thyroid function tests; TH, thyroid hormone; TSHR, TSH receptor; WT, wild type.

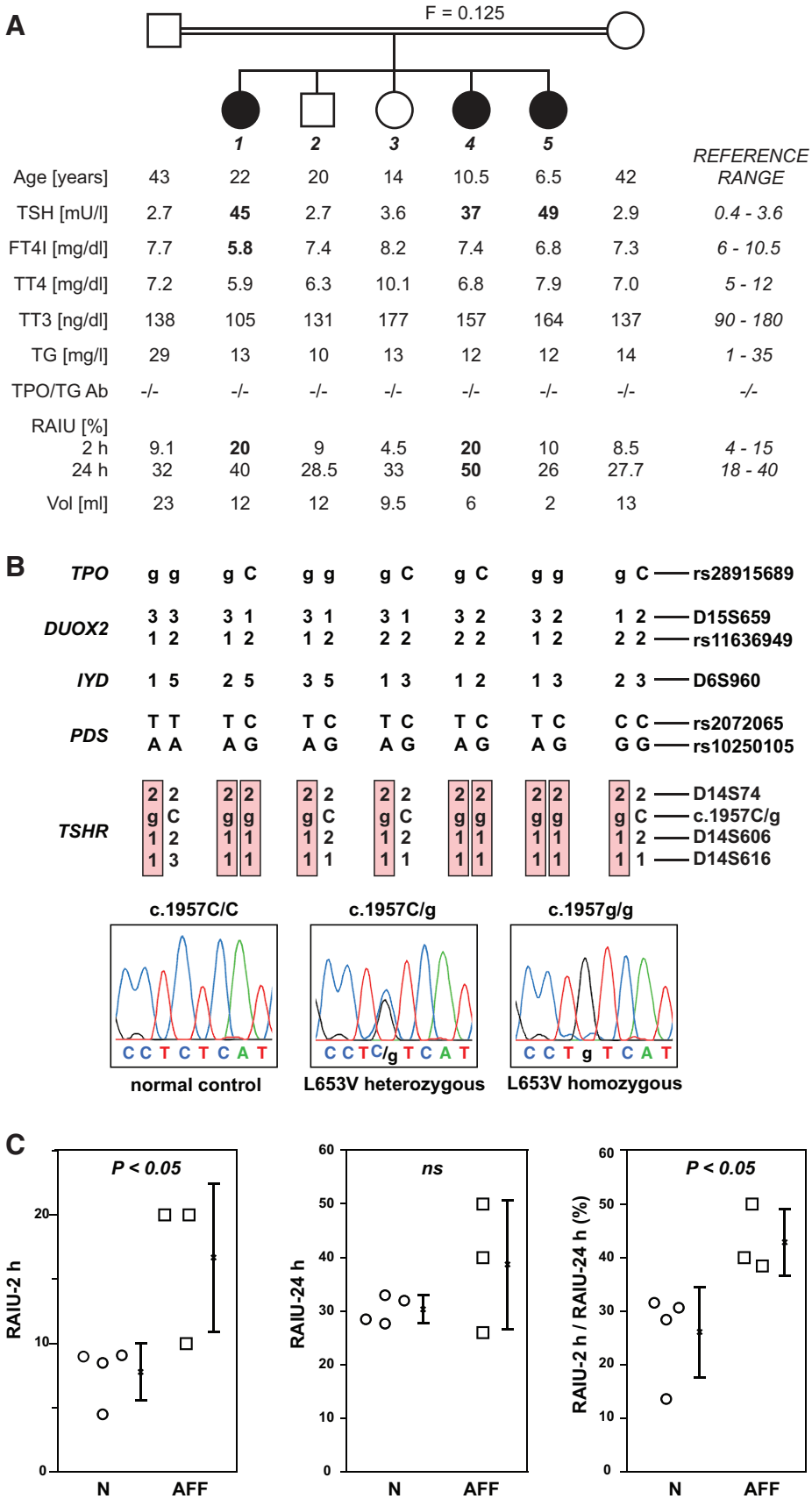

FIG. 1. Familial RTSH in three siblings. A, Results of TFTs are aligned with the symbols in the pedigree. Values outside the normal range are in *bold numbers*. F, Inbreeding coefficient; FT4I, free T_4 index; TT4, total T_4; TT3, total T_3; TG, serum thyroglobulin; TPO/TG Ab, antibodies against thyroperoxidase and thyroglobulin; Vol, thyroid gland volume by ultrasound. B, Genotyping results for the indicated markers are incompatible with linkage of the phenotype to *TPO*, *DUOX2*, *PDS*, or *IYD* but indicate homozygosity of affected, but not nonaffected, individuals at the *TSHR* locus. The sequencing electropherograms depict part of exon 10 of the *TSHR*. Shown are results for an unrelated control individual (*left*), the patients' mother (*middle*), and the affected subject 4 (*right*). The three patients were homozygous for a C to G transversion (c.1957C > G), resulting in replacement of leucine at position 653 by valine (L653V). The four other family members were heterozygous carriers of this mutation. C, Results of RAIU tests. Shown are the thyroidal uptake rates (percentage of the total dose) at 2 h (*left*) and 24 h (*middle*) after administration of ^{131}I and the ratio of the 2- to 24-h values (*right*). Affected subjects (AFF) have increased 2 h uptake and 2- to 24-h uptake ratios compared with unaffected (N) family members ($P < 0.05$; two-tailed *t* test). ns, Not significant.

but normal free and total TH levels. Antibodies against thyroperoxidase, thyroglobulin, and TSHR were undetectable. Her thyroid gland was in normal position. RAIU was 20% at 2 h (normal, 4–15) and 50% at 24 h (normal, 18–40). Her eutopic thyroid gland had normal size (6 cm^3) and echogenicity.

A younger sister (subject 5) (Fig. 1A) also had euthyroid hyperthyrotropinemia (TSH, 49 mU/liter; free T_4, 6.8 μg/dl). Her T_4 at birth was normal. The oldest sister (subject 1) was markedly hyperthyrotropinemic (TSH, 45 mU/liter). Both sisters had no signs or symptoms of hypothyroidism or hearing impairment. The parents and two other siblings had normal TFTs.

TFTs

TFTs were performed as described previously (7). RAIU measurements used a γ scintillation probe. Epithyroid counts were obtained at 2 and 24 h after 2 μCi of oral ^{131}I.

Linkage analysis and DNA sequencing

Studies were approved by the Institutional Review Boards of University of Chicago and Ha'Emek Medical Center. Written informed consents were obtained. Linkage analysis using microsatellite markers (D14S74, D14S606, and D14S616) spanning the *TSHR* locus was performed as described previously (7). Other markers assessing linkage were D6S960 [0.47 Mbp p-ter of *IYD* (*DEHAL1*)], D15S659 (0.96 Mbp q-ter of *DUOX2*), and intronic single nucleotide polymorphisms within *DUOX2*, *TPO*, and *PDS*. The complete *TSHR* coding sequence and intron-exon boundaries were sequenced bidirectionally. The c.1957C > G *TSHR* mutation was confirmed by *Mlu*I digestion.

Construction of expression vectors, cell culture, and transient transfection

The L653V TSHR expression vector was constructed by site-directed mutagenesis of the wild-type (WT) TSHR cloned in plasmid pSVL. COS-7 cells were cultured and transfected as reported previously (8).

Flow immunocytofluorometry

Cell surface expression of TSHR was quantified by flow immunocytofluorometry with monoclonal antibodies 3G4 (9).

Determination of cAMP production

cAMP was measured after 1-h incubation in Krebs-Ringer HEPES with 25 μM rolipram and various concentrations (0–100 U/liter) of bovine TSH (bTSH) (Sigma, St. Louis, MO) as described previously (8).

IP measurements

Cells labeled for 24 h with 20 μCi/ml [^{3}H]inositol were preincubated for 30 min with 10 mM LiCl and then were exposed for 15 min to various concentrations of bTSH. ^{3}H-labeled IPs were extracted and separated by stepwise anion exchange chromatography (3).

Homologous competitive TSH binding assay

Binding of [^{125}I]TSH (58 μCi/mg, 50–60 U/mg; Brahms Diagnostica, Berlin, Germany) to intact cells was determined in the presence of various concentrations of unlabeled bTSH (8).

Results

We identified three siblings with euthyroid hyperthyropinemia (Fig. 1A). All family members shared the same household in a nonendemic area in northern Israel. The high early (2 h) RAIU, indicating increased iodide trapping, prompted testing of all family members, revealing significantly higher 2-h uptake rates and 2- to 24-h uptake ratios in the three affected siblings compared with the normothyrotropinemic subjects (Fig. 1C). We hypothesized that a single recessively inherited defect could cause both euthyroid hyperthyrotropinemia and increased iodide trapping, a finding otherwise associated with defects in iodine organification or iodide deficiency.

Linkage of the phenotype to the *TSHR* locus was suggested because only affected individuals were homozygous for the same haplotype (Fig. 1B). Linkage to *DUOX2*, *TPO*, *PDS*, or *IYD* (*DEHAL1*) was excluded. All affected siblings carried a C to G transversion (c.1957C > G) on both *TSHR* alleles, producing a novel missense mutation (L653V) in the center of the third extracellular loop (ECL3) of the TSHR heptahelical domain. Both parents and the two nonaffected siblings were heterozygous for this mutation.

We assessed the function of L653V TSHR in transfected COS-7 cells. WT TSHR and P162A TSHR, which exhibits reduced TSH binding affinity (10), were analyzed in the same experiments. Results are summarized in Table 1, and representative experiments are displayed (Fig. 2). L653V TSHR was expressed at the cell surface at almost the same level as WT TSHR, determined in both TSH binding and flow cytometry studies, and had the same affinity for TSH as the WT TSHR (Fig. 2A). As indicated by a right shift of the concentration-response curves relative to those of WT TSHR (Fig. 2, B and C), the EC_{50} (for bTSH) of the L653V TSHR is higher for the cAMP signal (2.2-fold higher than WT EC50) and IP signal (2.2-fold), similar to the P162A TSHR (2.7-fold for cAMP and 2.8-fold for IP). Thus, the L653V and, as expected, the P162A mutation affect the potencies of bTSH for cAMP and IP induction equivalently. However, the most striking finding was the reduced efficacy of the L653V TSHR for IP

TABLE 1. Summary of the *in vitro* functional studies of the L653V TSHR

	[^{125}I]bTSH binding		Cell surface expression % of WT (mAb 3G4)	cAMP accumulation			IP accumulation		
				cAMP relative to WT basal			IP relative to WT basal		
	B_{max} (% of WT)	K_d (U/liter)		Basal	Stimulated (100 U/liter TSH)	EC_{50} TSH (U/liter)	Basal	Stimulated (100 U/liter TSH)	EC_{50} TSH (U/liter)
pSVL	(0)	n.d.	(0)	0.2 ± 0.02	0.3 ± 0.1	n.d.	1.2 ± 0.09	1.2 ± 0.1	n.d.
WT	100 ± 12.2	1.19 ± 0.35	100 ± 4.9	1 ± 0.03	5.7 ± 0.3	0.28 ± 0.06	1 ± 0.08	19.0 ± 2.6	6.9 ± 0.96
L653V	70.1 ± 5.2	1.13 ± 0.12	77.7 ± 6.0	0.95 ± 0.11	5.2 ± 0.5	0.63 ± 0.12	1.1 ± 0.08	3.7 ± 0.6	15.2 ± 3.0
P162A	26.3 ± 6.3	1.58 ± 0.46	78.8 ± 10.7	0.94 ± 0.06	5.9 ± 0.5	0.75 ± 0.10	1.1 ± 0.08	15.5 ± 2.1	19.4 ± 1.5

The TSH-binding parameters are represented as means ± SD values from five displacement curves. The results of cAMP and IP accumulation correspond to means ± SD values of four independent experiments each performed in duplicate transfections, except for the P162A control mutant, for which data from two experiments (each in duplicate transfections) were pooled and treated as independent experiments. The data for basal and stimulated second-messenger generation are expressed relative to the basal value (set to 1) in cells transfected with WT TSHR. pSVL, Cells transfected with empty vector; n.d., not determined.

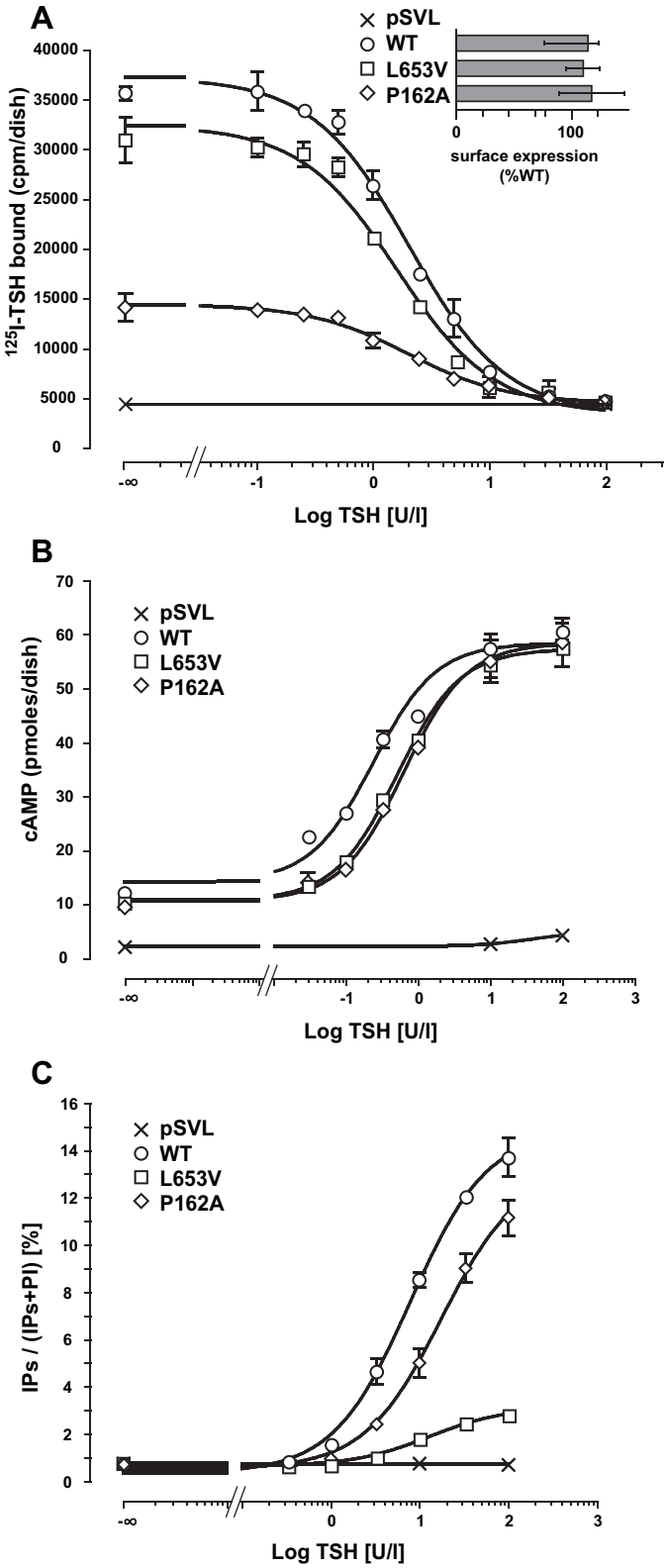

FIG. 2. Functional analysis of the L653V TSHR in transfected COS-7 cells. Characteristics of the L653V TSHR were compared with WT TSHR and the ligand-binding-deficient P162A TSHR. A, Homologous competitive binding assay. Results are from a single, representative experiment, performed in triplicate transfections. *Error bars* represent SD values. The corresponding analysis of surface expression by flow cytometry using 3G4 antibodies is shown in the *inset*. B, TSH-stimulated cAMP production. Shown are concentration-response curves from a single, representative experiment performed in duplicate transfections. *Error bars* indicate range of replicates. C, TSH-stimulated IP generation. Results are expressed as ratio (in percentage) of [^{3}H]inositol incorporation into IPs (*i.e.* IP_1, IP_2, and IP_3) to the sum of IPs and phosphatidylinositol. The concentration-response curves displayed are from one representative experiment. *Error bars* indicate range of replicates.

induction, measured as IP accumulation above pSVL-transfected cells at 100 U/liter bTSH. Efficacy for IP accumulation was only 14% of WT TSHR level. For comparison, the maximum effect of P162A TSHR (80% of WT) was compatible with its slightly reduced surface expression. In contrast to the mutation-specific effects on IP generation, the maximum cAMP response of WT and mutant TSHRs were essentially identical.

Discussion

Our data indicate that affected subjects have RTSH secondary to a homozygous loss-of-function mutation in the *TSHR* gene. L653V is the first natural missense mutation in ECL3 of TSHR that causes loss of receptor function. Surprisingly, affected subjects had significantly increased RAIU compared with unaffected members of the family, a clinical phenotype not described previously in TSHR loss-of-function mutations.

In human thyrocytes, the G_s/cAMP pathway controls positively cell proliferation, iodide uptake, and TH secretion, whereas the G_q/IP pathway stimulates iodination and, thus, TH synthesis (5, 11). Sodium iodide symporter expression is the most sensitive biomarker for stimulation of the cAMP cascade by TSH (12, 13). Increased iodide trapping in our patients implied that the elevated TSH levels overcompensate the defect in TSHR signaling. This also suggested that TH synthesis in the patients is limited by a defect in a cAMP-independent pathway, which drives the serum TSH above the level needed for compensation of the mild defect in cAMP signaling. This second pathway is likely an IP-triggered Ca^{2+} signal known to stimulate the generation of H_2O_2 crucial for all thyroid peroxidase-catalyzed reactions. We therefore hypothesized that the L653V mutation results in a "pathway-specific" defect, by causing a more severe defect in IP than in cAMP induction.

The importance of individual ECL3 residues for signal transduction was examined recently by a systematic alanine scan (14). Specifically, the L653A TSHR was shown to have essentially normal TSH binding affinity, slightly reduced cell surface expression (85% of WT TSHR), and reduced basal and stimulated cAMP accumulation in transfected COS-7 cells (62% of WT TSHR in stimulated cells). However, TSH-stimulated IP production of the L653A mutant was only 12% of WT TSHR. These data closely resemble our findings for the L653V mutation (Table 1). In addition, we have shown that L653V reduces the potency of TSH for cAMP and IP induction to essentially the same extent, whereas the imbalance in dual-pathway stimulation by the L653V mutant is attributable to diminished efficacy for IP generation.

These results can be explained by the emerging concept of TSHR activation based on an allosteric model (15) in which conversion of the ectodomain from a tethered inverse agonist

to an agonist (16) acts as a trigger, facilitating conversion into active conformations. The latter are likely distinguished by differential affinity to couple to G_s or G_q, as proposed for other G protein-coupled receptors interacting with distinct G protein classes and as demonstrated in TSHR-expressing CHO cells (17). In the framework of these models, L653V appears to have two effects that together account for its functional characteristics. First, L653V interferes with a trigger mechanism common to the G_s- and G_q-initiated pathways, as indicated by equivalently reduced potency for cAMP and IP induction similar to an upstream defect in ligand binding (P162A). This interpretation supports the important role of L653V in the intramolecular signal transduction mechanism, specifically in the agonist-like interaction of the ectodomain with the ECLs. Second, on activation of the trigger, L653V has a higher tendency, compared with WT TSHR, for transition into an allosteric state coupling to G_s, *i.e.* the L653V mutation disturbs the equilibrium between G_s- and G_q-coupled activated receptor states. Thus, an isomerization defect in a model with at least two active allosteric states can explain the drastically diminished efficacy of L653V TSHR for IP induction.

It should be emphasized that the substantially higher EC_{50} for IP *vs.* cAMP of WT TSHR *in vitro* are not caused by distinct potencies to couple to G_s and G_q but rather by differential efficiency of G proteins to activate their downstream effectors (1). G protein-effector coupling depends on cellular context and is modulated by external factors, *e.g.* by costimulation of G_i-coupled receptors, leading to sensitization of the IP and concomitant inhibition of the cAMP pathway (18). Thus, low sensitivity of the IP pathway for stimulation by TSH *in vitro* does not preclude a physiological role of this pathway in thyrocytes *in situ*.

Overall, our results revealed a specific TSHR defect, preferentially affecting the IP pathway, with a different phenotype than reported previously for TSHR inactivating mutations. This is the first *in vivo* evidence for the physiological role of the TSHR/IP/Ca^{2+} cascade in regulating iodination. The absence of spontaneous iodide discharge may reflect the limited role of this pathway in stimulating iodination, but one has to consider that the effect of the L653V mutation is neither absolutely specific for the IP pathway nor does it cause a complete loss of IP signaling. The results from *in vitro* mutagenesis studies indicate the potential for other TSHR mutations to cause similar or even more pronounced and specific IP signaling defects than the L653V mutation (14, 19, 20). Such TSHR mutations could underlie unexplained partial organification defects.

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Address all correspondence and requests for reprints to: Helmut Grasberger, The University of Chicago, MC3090, 5841 South Maryland Avenue, Chicago, Illinois 60637. E-mail: hgrasber@uchicago.edu.

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