0021-972X/97/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 82, No. 8
Printed in U.S.A.

Diversity and Prevalence of Somatic Mutations in the Thyrotropin Receptor and $G_s\alpha$ Genes as a Cause of Toxic Thyroid Adenomas*

JASMINE PARMA†, LAURENCE DUPREZ†‡, JACQUELINE VAN SANDE, JACQUES HERMANS, PIERRE ROCMANS, GUY VAN VLIET, SABINE COSTAGLIOLA, PATRICE RODIEN, JACQUES E. DUMONT AND GILBERT VASSART

Institut de Recherche Interdisciplinaire (J.P., L.D., J.V.S., S.C., P.R., J.E.D., G.V.); Department of Medical Genetics (J.P., G.V.), Université Libre de Bruxelles, Campus Erasme, 1070 Bruxelles; Centre Hospitalier de Jolimont (J.H.), Haine-Saint-Paul, Belgium; Service de Chirurgie Thoracique (P.R.), Hôpital Erasme, 1070 Bruxelles; Service d'Endocrinologie (G.V.V.), Hôpital Sainte-Justine, Montréal, H3T 1C5 Québec, Canada

ABSTRACT

A total of 33 different autonomous hot nodules from 31 patients, originating mainly from Belgium, were investigated for the presence of somatic mutations in the TSH receptor and $G_s\alpha$ genes. This constitutes an extension of our previous study, including the first 11 nodules of the series. The complete coding sequence of the TSH receptor gene and the segments of $G_s\alpha$ known to harbor mutations impairing guanosinetriphosphotase activity were studied by direct sequencing of genomic DNA extracted from the nodules. DNA from the juxtanodular tissue or peripheral white blood cells was analyzed in all patients to confirm that the mutations identified were somatic. Twenty-seven mutations (82%) were found in the TSH receptor gene, affecting a total of 12 different residues or locations. All these mutations but 2 (see below) have been identified previously as activating mutations. Only 2 mutations were found in $G_s\alpha$ (6%). In 4 nodules, no mutation was detected. Five residues (Ser281, Ile486, Ile568, Phe631, and Asp633) were found mutated in 3 or 4 different nodules, making them hot spots for activating mutations. Phe631 and Asp633 belong to a cluster of 5 consecutive residues (629–633) in the N-terminal half of transmembrane segment VI, which harbor together 44% of the mutations identified in this cohort. Two novel mutations were identified: a point mutation causing substitution of Phe for Leu at position 629 (L629F); and a deletion of 12 bases removing residues 658–661 at the C-terminal portion of exoloop 3 (del658–661). When tested by transfection in COS-7 cells, both mutant receptors display increase in constitutive stimulation of basal cAMP accumulation. Although it is still capable of binding TSH, the del658–661 mutant has completely lost the ability to respond to the stimulation by the hormone. Our results demonstrate that, in a cohort of patients from a moderately iodine deficient area, somatic mutations increasing the constitutive activity of the TSH receptor are the major cause of autonomous hot nodules. (*J Clin Endocrinol Metab* **82:** 2695–2701, 1997)

OVER THE past few years, it has become clear that mutations affecting the G protein-coupled receptors and $G_s\alpha$ genes constitute a pathophysiological explanation for a number of endocrine diseases (1). The known effects of TSH and cAMP agonists on thyroid cell function and growth (2), and knowledge about the structure-function relationships of G protein-coupled receptors (3) and the G proteins themselves (4), had provided the rationale to look for such mutations in autonomous thyroid adenomas (5–7).

In spite of rapid replication (8–10) of our initial observation of activating mutations in the TSH receptor (5), some authors have failed to identify similar mutations in a series of adenomas from Japan (9). Together with earlier claims that the TSH receptor did not behave as a proto-oncogene (11), this led to an uncertainty about the true prevalence of TSH receptor and $G_s\alpha$ mutations in toxic adenomas.

In the present study, we have completed our initial survey (5, 12, 13) by completely sequencing the coding portions of the TSH receptor gene from a total of 33 adenomas. In addition, the known hot spots for mutations affecting the GTPase activity of $G_s\alpha$ (14) were also sequenced. Twenty-seven mutations (82%) were found in a total of 12 different locations within the TSH receptor. These include 2 novel mutations that have been studied functionally. Only 2 mutations (6%) were identified in $G_s\alpha$. We conclude that in our population, somatic mutations of the TSH receptor gene are, by far, the most frequent cause of autonomous thyroid adenomas.

Materials and Methods

Patients

Twenty-two adenomas were added to a series of 11 investigated previously (12). In this earlier study, only the serpentine portion of the

Received January 22, 1997. Revision received March 21, 1997. Accepted April 29, 1997.

Address all correspondence and requests for reprints to: G. Vassart, I.R.I.B.H.N, Faculty of Medicine, University of Brussels, Campus Erasme, Route de Lennik 808, 1070 Brussels, Belgium. E-mail: gvassart@ulb.ac.be.

* This study was supported by the Belgian Programme on University Poles of Attraction initiated by the Belgian State, Prime Minister's office, Service for Sciences, Technology and Culture. The scientific responsibility is assumed by the author. This study was also supported by grants from the fonds de la Recherche Scientifque Médicale, The FNRS, Télévie, The European Union (Biomed), Association Belge contre le Cancer, and Association de Recherche Biomédicale et de Diagnostic.

† Contributed equally to this study.

‡ Aspirant at the Belgian FNRS.

TSH receptor had been sequenced, and the $G_s\alpha$ gene was not explored. Some characteristics of the patients and their adenomas are summarized in Table 1. All cases had a Tc-99 m scintigraphy. After surgery, the nodular and surrounding tissues were analyzed by standard pathology. A minority of cases presented with multinodular goiter with multiple zones of autonomy devoid of capsule. All patients had a suppressed plasma TSH.

DNA analyses

Genomic DNA was extracted from nodular and, when available, from juxtanodular tissue (if not, DNA was extracted also from peripheral white blood cells) (12). Care was taken to sample the zones identified as hot on the scintiscan. In some cases, the status of the sampled tissue was verified by ^{131}I uptake of tissue slices incubated *in vitro* (see Table 1) (15).

TSH receptor gene. All coding portions of the TSH receptor gene were sequenced using a total of 14 fragments amplified by PCR (the list of primers is available upon request). Direct genomic DNA sequencing of both strands was realized with the Thermosequenase sequencing kit (RPN 2436, Amersham Gent, Belgium) and mutations identified using the Factura and Sequence Navigator Software running on an ABI 373 sequencer.

$G_s\alpha$ gene. The exons 8 and 9 were amplified by PCR as previously described (16). The sequence reaction and analysis were performed as described above.

Functional analysis of novel TSH receptor mutants by transient transfection in COS-7 cells

Preparation of expression constructs. pSVL-TSHr constructs harboring the two new mutations were obtained as previously described (5, 17).

Transfections. COS-7 cells were grown and transfected as described (12). In brief, cells were seeded at the density of 300,000 cells/3-cm dish and transfected 1 day later with 500 ng/dish of each construct. Two days after transfection, cells were used for flow cytofluorometry, cAMP, or inositolphosphate determinations and ^{125}I-TSH-binding studies. Triplicate dishes were used for each measurement, except for inositolphosphate experiments, for which duplicates were used. Each experiment was repeated at least twice.

cAMP and inositolphosphate determinations. cAMP and inositolphosphate determinations were performed, essentially as described previously, by competitive binding assay and metabolic labeling, followed by ion exchange chromatography, respectively (12). For cAMP, results are expressed as picomoles per dish; for inositolphosphates, as the percentage of radioactivity incorporated from [^{3}H]-inositol in inositolphosphates (IP1 + IP2 + IP3) over the sum of radioactivity incorporated in inositolphosphates and phosphatidylinositols.

Binding assays. Binding assays were performed on whole cells incubated at room temperature for 4 h in NaCl-free Hank's buffer, supplemented with sucrose 280 (mmol/L), BSA (0.2%), and low fat milk (2.5%), exactly as described previously (18).

Flow cytofluorometry. Cells were detached from the plates with phosphate-buffered saline (PBS), supplemented with ethylenediamine tetraacetate (EDTA) and ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N'*-tetraacetic acid (EGTA) (5 mmol/L each), transferred into Falcon tubes (2052) and pelled by centrifugation at 500 × *g* for 3 min at 4 C. For the nonpermeabilized cell assay, cells were incubated immediately with the antibody. For the permeabilized cell assay, cells were first fixed in phosphate buffered saline-paraformaldehyde (PBS-PAF) 1% (Paraformaldehyde, UCB, Leuven, Belgium) for 10 min on ice and, thereafter, incubated with PBS-BSA 0.1%-Saponin 0.2% (Sigma Chemical Co., St. Louis, MO) for 30 min. Saponin-supplemented PBS buffer was used in all subsequent incubations. After incubation for 30 min at room temperature with 100 μL PBS-BSA (0.1%) containing the 2C11 mAb (10 μg/mL), the cells were washed with 4 mL PBS-BSA (0.1%), centrifuged

TABLE 1. Summary of some characteristics of the 33 nodules investigated in the present study (ND, not done)

Patient	Sex	Age at surgery	Origin	Nodule	*In vitro* ^{131}I trapping	TSHr Mutation	$G_s\alpha$ Mutation
1	F	57	JOLIMONT	MULTIPLE	ND	S281N	—
2	F	73	JOLIMONT	SOLITARY	ND	S281T	—
3	F	73	JOLIMONT	SOLITARY	ND	S281T	—
4a	F	59	JOLIMONT	MULTIPLE	ND	M453T	—
4b						T6321	—
5	M	35	BRUSSELS	SOLITARY	INCREASED	I486M	—
6	F	15	BRUSSELS	SOLITARY	INCREASED	I486F	—
7	M	77	JOLIMONT	SOLITARY	ND	I486F	—
8	F	23	BRUSSELS	SOLITARY	INCREASED	I568T	—
9	F	49	JOLIMONT	MULTIPLE	ND	I568T	—
10	M	45	JOLIMONT	SOLITARY	ND	I568T	—
11	F	16	MONTREAL	SOLITARY	ND	I568T	—
12	F	51	BRUSSELS	SOLITARY	INCREASED	D619G	—
13	M	60	BRUSSELS	SOLITARY	INCREASED	D619G	—
14	F	53	BRUSSELS	SOLITARY	INCREASED	A623I	—
15	M	60	JOLIMONT	SOLITARY	ND	L629F	—
16	M	64	JOLIMONT	SOLITARY	ND	I630L	—
17	F	59	BRUSSELS	ND	ND	F631L	—
18	F	50	BRUSSELS	SOLITARY	INCREASED	F631L	—
19	F	14	BRUSSELS	SOLITARY	INCREASED	F631L	—
20	F	68	JOLIMONT	SOLITARY	ND	F631L	—
21a	M	32	JOLIMONT	MULTIPLE	ND	T632I	—
21b						—	—
22	F	57	JOLIMONT	SOLITARY	ND	D633A	—
23	F	73	JOLIMONT	MULTIPLE	ND	D633E	—
24	F	27	JOLIMONT	SOLITARY	ND	D633H	—
25	M	55	JOLIMONT	SOLITARY	ND	D633Y	—
26	F	63	JOLIMONT	SOLITARY	ND	del658–661	—
27	F	75	JOLIMONT	SOLITARY	ND	—	R201C
28	F	53	JOLIMONT	SOLITARY	ND	—	R201H
29	M	62	BRUSSELS	SOLITARY	INCREASED	—	—
30	F	52	BRUSSELS	ND	ND	—	—
31	F	73	JOLIMONT	MULTIPLE	ND	—	—

as above, and incubated for 30 min on ice in the dark with fluorescein-conjugated γ chain-specific goat antimouse IgG (Sigma) in the same buffer. The 2C11 mAb, kindly provided by Dr. A.P. Johnstone (19), recognizes a linear epitope of the extracellular aminoterminus of the TSH receptor (VFFEEQ, residues 354–359)(20). Propidium iodide (10 μg/mL) was used for detection of damaged cells, which were excluded from the analysis. Cells were washed once again and resuspended in 250 μL PBS-BSA (0.1%). The fluorescence of 10,000 cells per tube was assayed by a FACScan Flow Cytofluorometer (Beckton Dickinson, San Jose, CA).

Results

Relative frequency of TSH receptor and $G_s\alpha$ mutations

In our initial studies (5, 12, 13), 11 patients were investigated for the presence of somatic mutations within the serpentine portion of the TSH receptor encoded by exon 10 of the gene. We have now completed this series by studying 20 additional patients, yielding a total of 33 adenomas (2 patients harbored 2 separate hot nodules). To avoid the sampling bias of previous studies, the full coding portion of the TSH receptor gene was sequenced, including the 9 exons encoding the aminoterminal extracellular domain (5, 8, 9, 12, 13, 21, 22). Also, the 2 hot spots for mutations in $G_s\alpha$ were explored (residues 201 and 227) (14). The results are illustrated in Table 1, together with some characteristics of the patients and their tumors. A somatic mutation of the TSH receptor gene was found in 27 adenomas (82%, confidence interval 95%: 65–93), whereas only 2 were found in $G_s\alpha$ (6%, confidence interval 95%: 1–20). In all cases, the mutations were confined to the adenomatous tissue. As reported previously, 1 patient with a multinodular goiter had 2 hot nodules with a different mutation in each (23).

Spectrum of mutations in the TSH receptor

The 27 mutations identified involve a total of 12 different locations in the TSH receptor gene (Fig. 1), some regions or residues constituting convincing hot spots (Fig. 1). The 6th transmembrane segment alone harbors 12 of the 27 mutations (44%). Detailed inspection of the various mutations revealed no obvious bias in base substitutions (Fig. 1). Specifically, no single mutation affected CpG dinucleotides that are known as hot spots for mutations in vertebrates. However, the diversity of mutations at different positions vary considerably: whereas a variety of aminoacid substitutions are observed at some positions (*e.g.* Asp633, Ser281), the same substitutions are repeatedly observed at others (*e.g.* Ile568, Phe631). This suggests strongly that selection of the mutations is based essentially on functional criteria, *i.e.* their aptitude to trigger proliferation of (hyper)functional thyrocytes (see *Discussion*).

Functional characterization of 2 novel activating mutations

Except for 2 (L629F and del658–661), all mutations have been documented and analyzed functionally by transfection in COS-7 cells (5, 8, 12, 21, 22, 24–26). Whereas L629F is included in the major hot spot area at the basis of transmembrane VI, del658–661 is unusual both in its location (the border between the 3rd exoloop and transmembrane segment VII) and its nature (a deletion of 12 bases, removing 4 aminoacids). The mutations are illustrated in Fig. 2.

When transfected in COS-7 cells, L629F behaves as most other activating mutants described previously: it causes an increase in basal cAMP accumulation, as compared with the wild-type receptor (Fig. 3, panel a), does not affect the basal level of inositolphosphates (Fig. 3, panel b), continues to

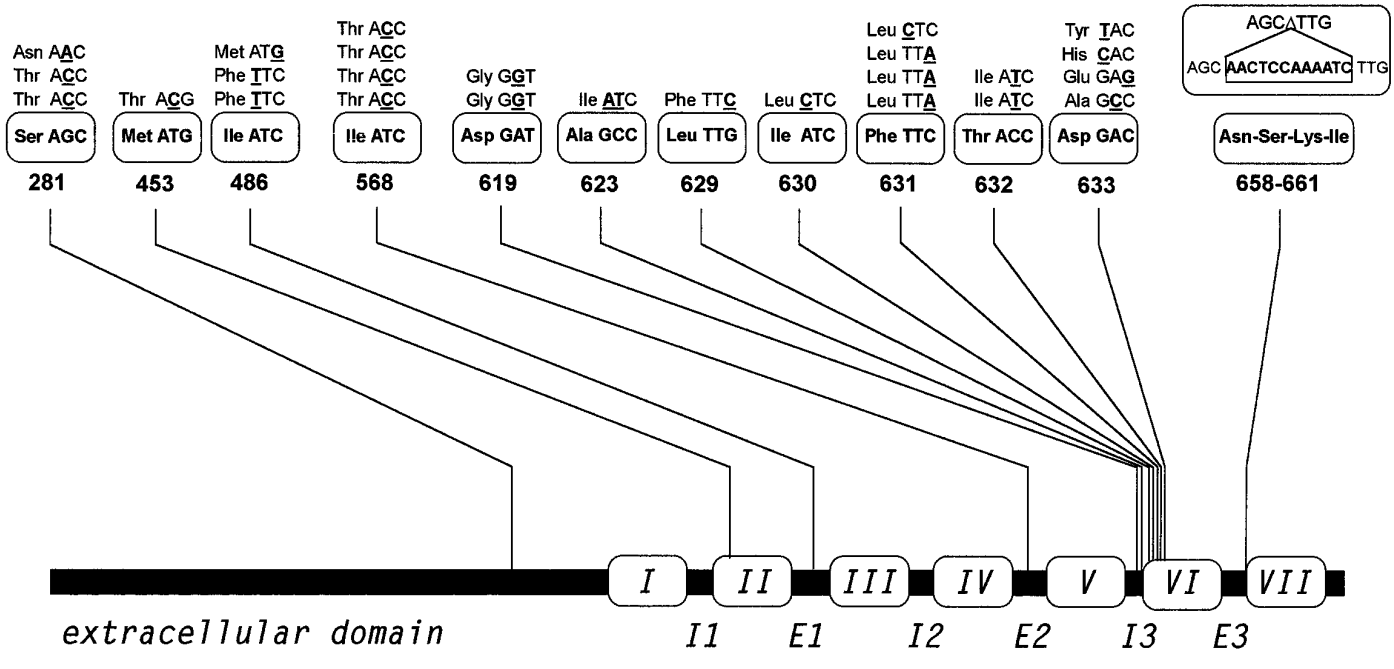

FIG. 1. Schematic representation of the TSH receptor, with indication of the residues found mutated in a cohort of 31 patients with a total of 33 toxic adenomas (in 2 patients, 2 distinct hot nodules were investigated). Every individual substitution is indicated. The transmembrane segments are represented by *boxes* (I to VII); the extra- and intracellular loops are indicated by E1, 2, and 3 and I1, 2, and 3, respectively.

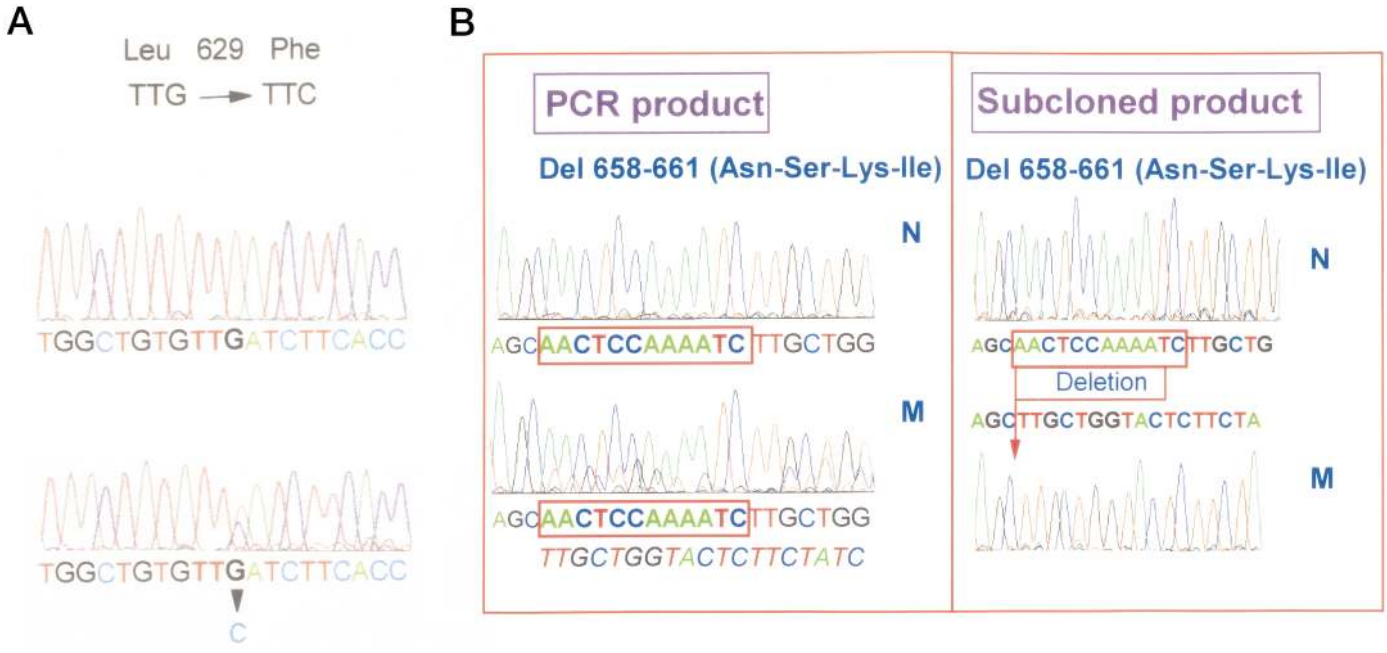

FIG. 2. Panel a, Direct sequencing of the PCR product from the adenoma in patient no. 15 (see Table 1) is shown, displaying substitution of a Phe (TTC) for Leu (TTG) at position 629; panel b, direct sequencing of the PCR and the subcloned products from the adenoma in patient no. 26, displaying deletion of 12 bases encoding Asn-Ser-Lys-Ile (residues 658–661). In both cases, coding strands are shown for the quiescent tissue harboring the normal alleles (N) and for the nodule harboring the mutated allele in the heterozygous state (M): mutated *vs.* normal allele ratio is lower than 1.

respond to TSH for both cAMP and inositolphosphate accumulation (Fig. 3 and Fig. 4, panel a), and binds TSH with slightly higher affinity than the wild-type (Fig. 4, panel b). Expression of L629F mutant at the cell surface is lower than the wild-type (Fig. 5).

The deletion mutant displays a more interesting phenotype. Whereas it causes larger increase of basal cAMP accumulation than L629F (Fig. 3, panel a), its expression at the cell surface is much lower (Fig. 5). Most of the mutated receptor molecules remain trapped intracellularly, as revealed by flow immunocytometry of cells permeabilized with saponin (Fig. 5). Similar to L629F, del658–661 has no effect on basal inositolphosphate accumulation (Fig. 3, panel b), but contrary to L629F, it has completely lost the ability to respond to TSH for stimulation of both adenylyl-cyclase and phospholipase C (Fig. 3). Binding of labeled TSH is clearly present (Fig. 4, panel b), but it is difficult to analyze quantitatively, because of the low level of expression at the plasma membrane.

Discussion

Our results identify somatic mutations of the TSH receptor gene in the majority of the toxic thyroid adenomas that we have studied. Mutations in $G_s\alpha$ account for a minority of cases only. Considering the known role of cAMP agonists on thyrocyte growth and function (27, 28), and in agreement with transgenic mice models (29, 30), these mutations that enhance the basal adenylyl-cyclase activity in thyrocytes provide a convincing pathophysiological explanation for the development of thyroid autonomy. It must be emphasized, however, that additional mutational events might be implicated in the continuous growth of toxic adenoma, as suggested by experimental data demonstrating a limitation in the growth potential of normal thyrocytes when stimulated continuously by TSH or cAMP agonists (31, 32).

The large excess of TSH receptor mutations (82%) over $G_s\alpha$ mutations (6%) may have more than one explanation: first, as demonstrated by transgenic mice models, constitutive activation of the adenylyl-cyclase/cAMP pathway is more efficient at the receptor level than at $G_s\alpha$ (29, 32); second, as our results demonstrate, the number of effective targets for activating mutations is much higher in the TSH receptor (at least 12 different locations, see Table 1) than in $G_s\alpha$ (2 residues) (14).

Studies from other groups do not all agree with the prevalence of TSH receptor mutations observed here. In a Japanese study, no activating mutation could be found in 38 toxic nodules (9); in 1 Italian study, activating mutations were observed in 7 of a series of 11 toxic adenomas (8); in another, 37 hot nodules were found to harbor 3 and 9 mutations in the TSH receptor and $G_s\alpha$, respectively (22). This may have methodological causes (direct sequencing was not always used; only selected segments of the receptor gene were studied), but it may reflect, in part, a true difference in the underlying pathophysiological mechanism. The chronic low-grade stimulation of the thyroid associated with the mild iodine deficiency of our population may favor the accumulation of somatic mutations, as discussed in reference to the case displaying different mutations in his 2 hot nodules (Nr4 in Table 1) (23). That (an)other pathophysiological mechanism(s) may be implicated is suggested by our observation that, in 4 nodules out of 33 (12%), we were unable to identify any mutation in the 2 genes. This may reflect limitation in our methodology, pitfalls in sampling, mutations in other genes

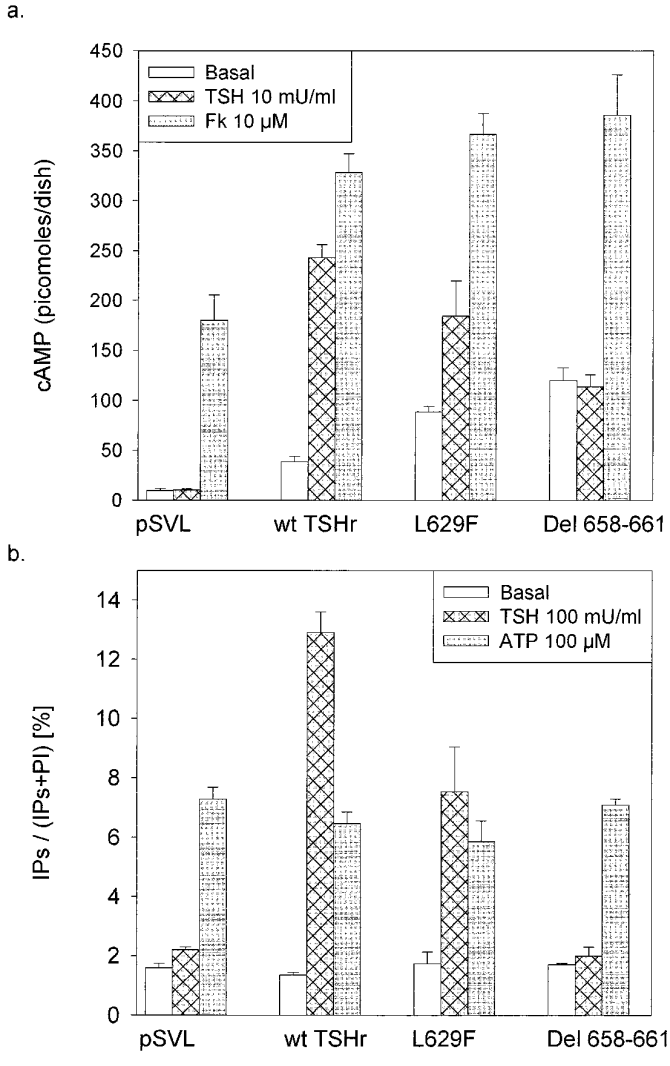

FIG. 3. Functional characteristics of the two novel TSH receptor mutants analyzed by transfection in COS-7 cells. Panel a, Effects on the cAMP production. Basal, TSH- or forskolin-stimulated cAMP levels are shown in cells transfected with the mutants in comparison with the wild-type receptor. Results are from one representative experiment out of six. Panel b: Effects on inositolphosphate production. Basal, TSH- or ATP-stimulated inositolphosphate accumulation was measured in cells transfected with the mutants in comparison with the wild-type receptor, and results are expressed as described in *Materials and Methods*. Values are from one representative experiment out of three.

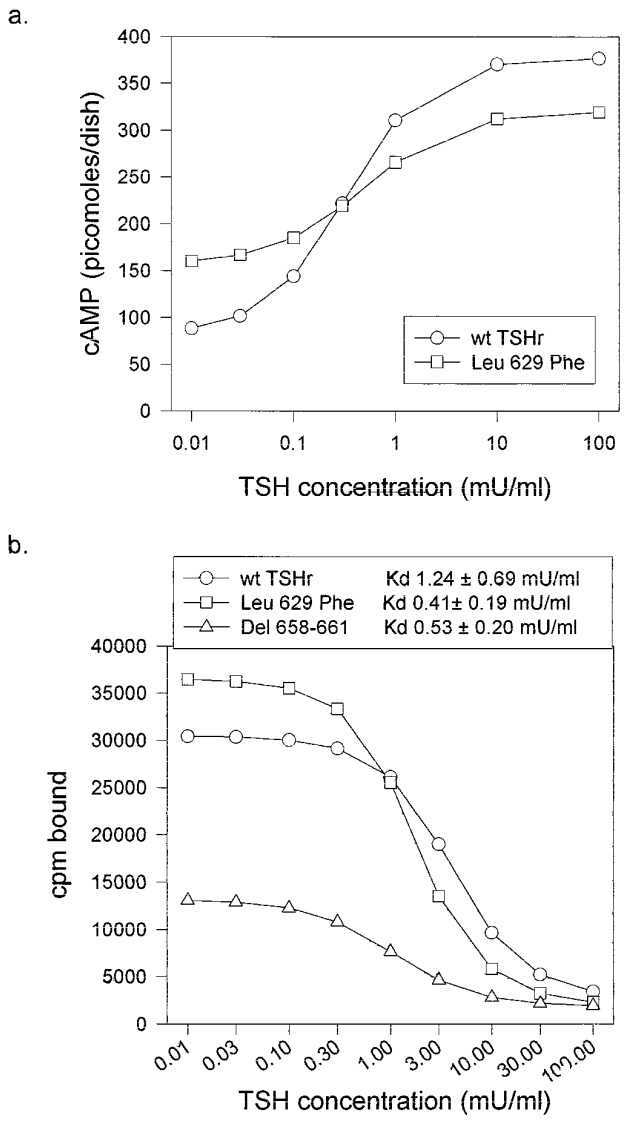

FIG. 4. Panel a, TSH stimulation of cAMP accumulation in COS-7 cells transfected with the wild-type receptor or the Leu629Phe mutant. Panel b, Competition binding curves of ^{125}I-TSH on COS-7 cells expressing the wild-type receptor and the Leu629Phe and the del658–661 mutants. Illustrative binding curves (see *Materials and Methods*) are from one representative experiment out of four. K_d is expressed in mU/mL (mean ± SD; n = 4).

or in regions controlling the level expression of the TSH receptor gene (33), or nonmutational epigenetic mechanisms.

The 27 mutations of the TSH receptor identified in this cohort involve 12 different locations or residues. Together with somatic mutations identified by other groups (8, 22) and germline mutations responsible for hereditary or sporadic toxic thyroid hyperplasia (18, 24, 25, 34), the total number of targets for activating mutations in the TSH receptor is currently 19. This surprising diversity of activating mutations tells us that activation of the TSH receptor is most likely a result of the release of an inhibitory structural constraint (35, 36). In the absence of the agonist, the equilibrium between inactive and active conformations of the receptor could thus be shifted toward active conformations by a wide spectrum of different alterations in the primary structure, having similar or equivalent effects on the tertiary structure. With this model in mind, it is interesting to explore the spectrum of amino acid substitutions at the different positions where activating mutations are observed. If one considers only the mutations observed in this cohort, residues can be classified into two categories: those, like Ile568 and Phe631, where the same aminoacid substitutions are repeatedly observed (Ile568Thr in 4/4 cases; Phe631Leu in 4/4 cases); others, like Asp633, which can be changed to a variety of aminoacids belonging to different classes (Glu, Tyr, His, Ala). This could mean that, depending on the residue, some mutations might destroy interactions stabilizing the inactive conformation of

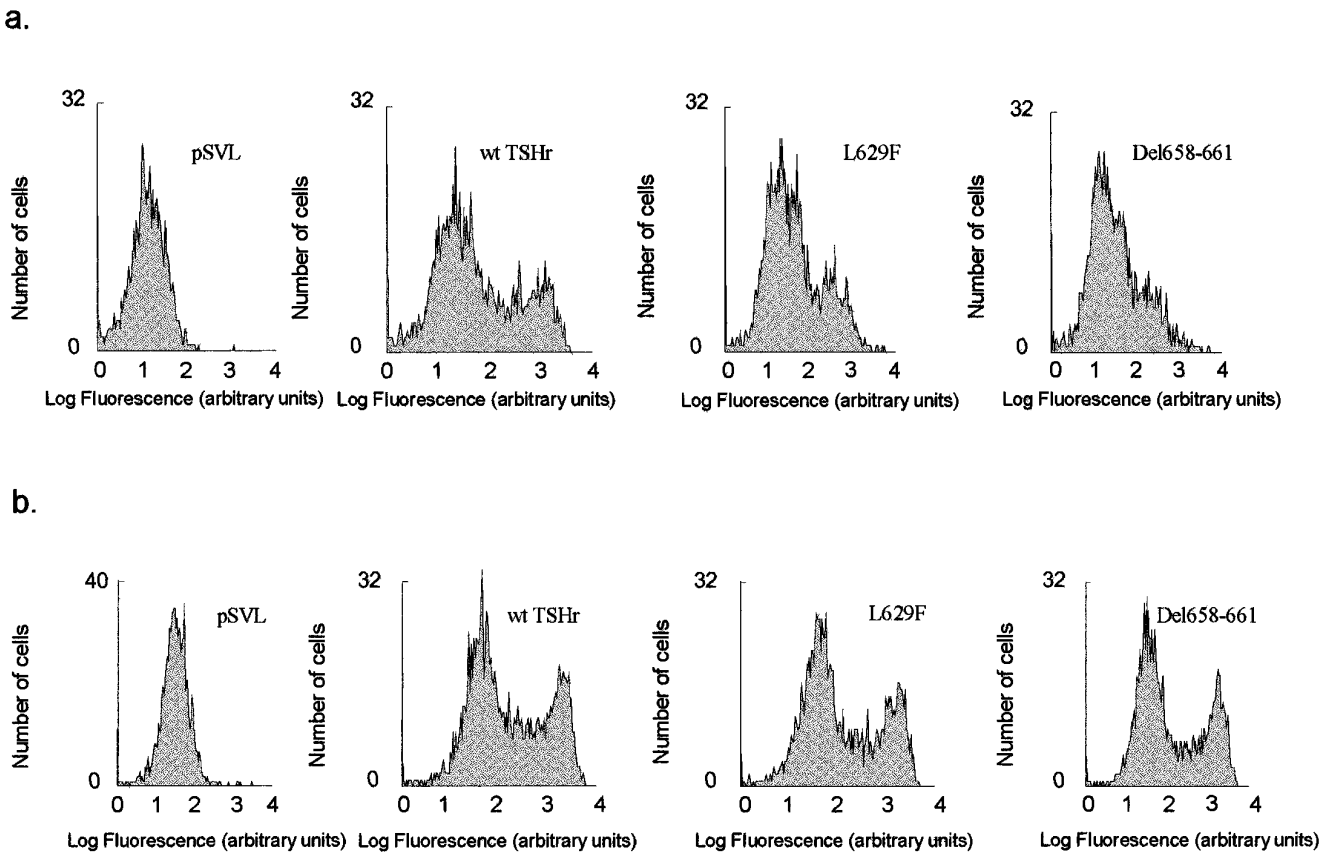

FIG. 5. Level of expression of the novel mutant receptors, as measured by flow cytofluorometry. COS-7 cells transfected with the empty pSVL vector, the wild-type constuct, or the two mutants were subjected to flow immunofluorometry using the 2C11 monoclonal antibody. Panel a, Expression at the surface of intact cells; panel b, total expression as measured from cells permeabilized with 1% saponin. For details, see *Materials and Methods*.

the receptor, whereas others would involve the establishment of new attractive or repulsive interactions. However, because we cannot predict which substitutions would prevent receptor expression at the plasma membrane (37, 38), it can simply be that the identical substitutions observed repeatedly at some residues would result from a selection bias (all other substitutions at these residues being either silent, or incompatible with correct receptor expression).

Two novel mutations have been identified: the first, L629F, can be added to the list of mutations affecting the hot spot in transmembrane VI. Their functional characteristics do not deviate from the average mutations identified in this region. Transmembrane VI is considered to play a key role in the transmission of an activation signal to the G protein by altering the conformation or position of the third cytoplasmic loop (39). The second mutation, del658–661, is more interesting, both from its nature and functional characteristics. To the best of our knowledge, it is the first natural activating mutation caused by a deletion. Although it is poorly expressed at the surface of COS-7 cells (Fig. 5), it is the first to display strong constitutive activity while having lost completely the ability to respond to TSH (Fig. 3). In keeping with the discussion above, it is likely that this deletion would destroy an interaction and/or, from its location at the C-terminal portion of the third exoloop, modify the position of transmembrane segments VI or VII. It is tempting to explain the functional characteristics of this mutant in the light of a model in which interactions between the aminoterminal domain and the exoloops of the receptor would be implicated in maintaining the unliganded receptor in an inactive conformation (20).

Comparing the spectrum of somatic mutations found in toxic adenomas with germline mutations found in hereditary toxic thyroid hyperplasia, leads to the surprising observation that they do not overlap (18, 34, and the present report). A likely explanation is that this reflects a selection bias. Starting from a single cell, to produce a sizable tumor, the adenoma type must be more aggressive to be selected by the clinical screening. In comparison, mutations causing a milder stimulation are expected to cause hyperthyroidism when they are expressed in all thyrocytes, as in the familial diseases. When occurring in the germline, mutations of the adenoma type indeed cause severe congenital hyperthyroidism (24, 25) and are expected to significantly decrease reproductive fitness in the absence of treatment.

Apart from this gross subdivision between hereditary and somatic or neomutations, there is no obvious correlation between the nature of the mutations in toxic adenomas and the clinical picture. Even the TSH receptor mutations identified in rare cases of thyroid cancers all belong to the spectrum of mutations found also in benign toxic adenomas (40, 41).

Acknowledgments

We express our gratitude to the clinicians who contributed to the study: Jean Mockel, Guy Andry, and Nelly Mirkine. Viviane De Martelaere and Christiane Christophe helped with the statistics and the synthesis of the primers, respectively. The expert technical assistance of Muriel Nguyen and Marie-Jeanne Simons is greatly acknowledged. The 2C11 monoclonal antibody is a kind gift of Dr. A. Johnstone (London), and the ^{125}I TSH tracer was provided by B.R.A.H.M.S. Diagnostica (Berlin).

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