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# Mutation of Somatostatin Receptor Type 5 in an Acromegalic Patient Resistant to Somatostatin Analog Treatment

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**Introduction of somatostatin analogs has greatly contributed to improving the prognosis of acromegaly. Although the majority of patients are effectively treated by these agents, resistance occurs in a subset of patients. So far, resistance to somatostatin has never been associated with mutations of the somatostatin receptor subtypes (sst2 and sst5) that inhibit GH secretion. Molecular analysis of genomic DNA from pituitary tumor and peripheral blood obtained from an acromegalic resistant to octreotide showed a somatic activating mutation of Gsα (Arg201Cys), no mutation in sst2, and one polymorphism (Pro109Ser) and one germ line mutation (Arg240Trp) in sst5. Wild-type (WT) and mutant sst5 PCR products were cloned and transfected into Chinese hamster ovary K1 cells. In Chinese hamster ovary K1 cells stably expressing mutant sst5, somatostatin-28 was less potent in inhibiting cyclic AMP levels than in WT cells. Proliferation of mutant cells exceeded that of WT by 50%. Moreover, somatostatin reduced cell growth and MAPK activity in WT but not in mutant cells in which the peptide even increased MAPK activity. We suggest that this mutation that abrogates the antiproliferative action of somatostatin and activates mitogenic pathways may be involved in the resistance to somatostatin treatment. (*J Clin Endocrinol Metab* 86: 3809–3814, 2001)**

ACROMEGALY IS A chronic disease characterized by elevated GH and IGF-I levels, generally owing to a GH-secreting pituitary adenoma. When inadequately treated, acromegaly results in significant morbidity and greatly increased mortality (1–3). Because only about 50% of patients are effectively cured by the surgical removal of the adenoma (4, 5), medical therapy has an important role in the management of these patients (6, 7). The introduction of new drugs, especially somatostatin analogs, has greatly contributed to improve the prognosis of acromegaly (6–10).

Somatostatin is the hypothalamic peptide that physiologically inhibits GH secretion. The peptide binds to a family of five specific G protein-coupled receptor subtypes (sst1–5) with important differences in tissue distribution, coupling to second messengers, and affinity for somatostatin and its analogs (11–13). The commercially available long-acting somatostatin analogs, such as octreotide and lanreotide, bind with high affinity to sst2, sst5, and to a lesser extent sst3 (11–13). Activation of these receptors generates a series of intracellular events, including inhibition of adenylyl cyclase and reduction of cytosolic calcium (11, 13). Moreover, somatostatin exerts direct antiproliferative effects on cell lines by triggering still not completely defined signals (10, 13–19). In particular, in cells expressing sst2 and sst5, somatostatin reduces the activity of MAPK, a family of protein serine/threonine kinases considered key molecules in transducing growth factor proliferative signals (13, 17, 18).

Abbreviations: CHO, Chinese hamster ovary; GAPDH, glyceraldehyde phosphate dehydrogenase; sst, somatostatin receptor subtype; WT, wild-type.

Somatostatin analogs lower GH levels in about 90% of acromegalic patients, although IGF-I and GH levels low enough to be considered safe in respect to normalization of life expectancy are achieved in only about 60% of patients (7–10). In addition, somatostatin analogs block the growth of the tumoral mass and may indeed slightly reduce tumor size in up to 50% of patients (10). The molecular basis of the different sensitivity to somatostatin remains largely unknown. There is a general agreement that patients harboring tumors with mutations of the Gsα gene that constitutively activate adenylyl cyclase (referred to as *gsp* mutations) (20) are highly sensitive to the inhibitory action of somatostatin (21, 22). Conversely, because no mutations of sst2 and sst5 genes have so far been reported (23), resistance has been attributed to a reduced expression of sst2 or sst5 (24–26).

In the present study, we report the first mutation of the sst5 gene that abrogates the antiproliferative action of somatostatin and activates mitogenic pathways in one acromegalic patient resistant to somatostatin analog octreotide and carrying *gsp* oncogene.

## *Case report*

The patient, a 49-yr-old women, was admitted to our clinic in 1997 because of signs and symptoms of acromegaly that was confirmed by hormonal data (Table 1). Magnetic resonance imaging revealed a 10-mm pituitary adenoma. The patient underwent pituitary surgery with only partial removal of the tumor. The patient was then treated with the depot long-acting somatostatin analog octreotide LAR 20 mg every 28 d for 6 months and then increased to 30 mg every

**TABLE 1.** Hormonal data of the acromegalic patient resistant to somatostatin treatment

| | GH[a] μg/liter | IGF-1[b] nmol/liter | Insulin[c] μU/mliter |
|---|---|---|---|
| At diagnosis, fasting[a] | 9.0 | 128 | 8.4 |
| At diagnosis, OGTT[d] | 6.8 | | |
| After surgery, fasting[a] | 6.2 | 84 | 6.1 |
| After surgery, OGTT[d] | 7.6 | | |
| Octreotide LAR 20 mg, fasting[a] | 6.3 | 80 | 7.2 |
| Octreotide LAR 30 mg, fasting[a] | 6.5 | 114 | 6.1 |
| Cabergoline, fasting[a] | 4.1 | 48 | 9.4 |

To convert values for serum IGF-I to ng/ml, multiply by 7.7; to convert values for serum insulin to pmol/l, multiply by 7.1.

[a] GH fasting: mean of three samples taken in 2 h; required value < 2 μg/l.

[b] IGF-I: normal range for age: 12.6–38.2 nmol/l.

[c] insulin fasting: normal range: 5–20 μU/ml.

[d] GH oral glucose tolerance test: nadir concentration during glucose load; required value < 1 μg/l.

OGTT, Oral glucose tolerance test.

28 d for another 6 months. During this period multiple hormonal determinations according to standard protocols revealed no modifications of GH and IGF-I levels (Table 1). Also, serum insulin, whose concentrations are known to be reduced by somatostatin analogs, did not change during treatment, both in fasting state (Table 1) and after oral glucose tolerance test (3-h AUC: before therapy 5196 *vs.* 4743 μU/min per milliliter during therapy). After 6 months of therapy, no shrinkage of the tumor remnant was observed at magnetic resonance imaging. Thus, octreotide therapy was interrupted, and the patient was then given cabergoline, a long-acting dopaminergic agonist, that caused a clear reduction of GH and IGF-I levels after 6 months at the dose of 3 mg/wk. The patient is actually under dopaminergic treatment. Written informed consent was obtained from the patient after the study had been approved by the local ethical committee.

## Materials and Methods

### *DNA and RNA extraction and sequencing*

Genomic DNA and RNA were extracted according to standard procedures, as previously described (27). The sst2 and sst5 genes were amplified by PCR using the following primers pairs: 5′-CTGGCTGGAACTAGCCTAAGACT-3′ and 5′-GACAGAGCATGGCTTGGTTCTTT-3′, 5′-TCTTCTCTTGCAGAGCCTGA-3′ and 5′-CGCAGTGCAACCTCCGCTCCTGGGGGTGA-3′, respectively. These primers identified sequences of sst2 and sst5 genes of 1184 and 1194 bp, respectively. A 50-μl reaction mix (0.5 μg of DNA sample, 50 nmol/l KCl, 50 nmol/l Tris-KCl (pH 8.3), 1 nmol/l MgCl2, 25 pmol of each primer, and 2.5 U *Taq* DNA polymerase AmpliTaq (Perkin-Elmer Corp., Foster City, CA) was subjected to denaturation at 94 for 3 min, followed by 35 cycles of 94 C for 60 s, 62 C for 60 s, and 72 C for 1 min. A final cycle at 72 C for 10 min was carried out to allow complete extension of the amplified fragments. The PCR products were sequenced from both strands with an automatic technique. Total RNA was reverse transcribed and PCR performed on the entire cDNA product with *Taq* DNA polymerase and specific primers for sst5. Genomic DNA from the tumor was analyzed for the presence of *gsp* mutations by amplifying the hot spots of the Gsα gene, as previously described (27).

### *Cloning and transfection of the human sst5 gene*

WT and mutant sst5 PCR products were directly cloned using the Eukaryotic TA cloning kit (Invitrogen, Carlsbad, CA) in two mammalian expression vectors pCR3.1. The two resulting vectors were separately transfected into Chinese hamster ovary (CHO) cells (CHOK1 strain) using the calcium phosphate precipitation method. Stably transfected cells were selected in modified Eagle's medium (αMEM) with 10% FCS using geneticin 500 μg/ml (Sigma, St. Louis, MO) and surviving colonies were subcloned and expanded. For each vector transfected, three clones expressing the WT (WTsst5/15, WTsst5/20, and WTsst5/25) and mutant (R240Wsst5/6, R240Wsst5/26, and R240Wsst5/27) receptors were selected by binding studies. CHOK1 cells were concomitantly transfected with the vector devoid of sst5 and used as control clone.

### *Semiquantitative determination of sst2 and sst5 gene transcripts*

Total RNA was extracted from the tumoral tissue and the levels of sst5 RNA transcripts were evaluated by semiquantitative RT-PCR using appropriately selected primer pairs, as previously described (27). The sequences of the oligonucleotides used for the amplification of sst2 and sst5 genes are available on request to the author. Data were compared with those of eight GH-secreting adenomas removed from patients responsive to octreotide. The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as internal standard. Preliminary experiments were conducted to determine the PCR cycles corresponding to the exponential phase for both genes. Results were expressed as the ratio of the radioactivity of each fragment for sst genes to that of GAPDH.

### *Membrane preparation and binding studies*

For membrane preparation, CHOK1 cells were grown in 10-cm dishes, and binding studies were performed on cell lysates as previously described, with minor modifications (28). Briefly, membrane preparations (20 μg/tube) resuspended in 50-mM HEPES, 0.1 mM CaCl2, 10 mM MgCl2, protease inhibitors, and 0.5% BSA were incubated with (125ITyr11) somatostatin-14 (Amersham Pharmacia Biotech, Aylesbury, United Kingdom) at 25 C for 120 min with and without 1 μM somatostatin-28 (Sigma) in triplicate. Bound ligand was trapped by vacuum filtration with a Millipore Corp. (Bedford, MA) cell harvester through Watman GF/C filters (Clifton, NJ). The filters were immediately washed with a 15-ml buffer and then counted on a γ-counter.

### *cAMP measurement*

For intracellular cAMP assay, CHO cells (at $8 \times 10^5$ in 6-well plastic plates) were preincubated with isotonic HBSS (Sigma) containing 0.4% BSA and 0.5 mM 3-isobutyl-1-methylxanthine, washed, and subsequently exposed for 30 min at 37 C to 1 μM forskolin with or without somatostatin-28. Cells were extracted in cold ethanol after 12–24 h at −20 C, lyophilized, and assayed by enzymatic immunoassay (Amersham Pharmacia Biotech) as previously described (29).

### *Cell growth assay*

CHO ($1 \times 10^5$) were cultured in αMEM containing 10% FCS and plated in 35-mm dishes. After 24-, 48-, and 72-h incubation, the number of WT or mutant cells was evaluated using a Coulter counter. To study the effect of somatostatin on cell growth, cells were cultured in αMEM containing 10% FCS with or without 10 nM octreotide (provided by Novartis Spa, Basel, Switzerland) and counted after 24-h incubation.

### *Western blot analysis of phospho-44/42- MAPK and MAPK activity*

Cells ($1 \times 10^6$) serum starved for 18 h were incubated with αMEM containing 10% FCS with or without 1 nM somatostatin-28 for 10 min. Lysates were collected in the presence of protease and phosphatase inhibitors and protein concentrations determined by BCA protein assay kit (PierceChemical, Rockford, IL). Western blot of phospho-44/42-MAPK was performed using an anti-phospho-44/42- MAPK (ERK-1/2) polyclonal antibody diluted 1:1000 (New England Biolabs, Inc., Beverly, MA) and detected by chemiluminescent method. MAPK activity was measured on lysates immunoprecipitated with anti-phospho-44/42MAPK antibody and incubated with Elk-1 fusion protein and kinase buffer for 30 min. Western blot was performed using anti-phospho-Elk1

antibody. Bands were evaluated by an imaging densitometer (GS-700, Bio-Rad Laboratories, Inc., Richmond, CA).

### *Statistics*

The results are presented as the mean ± SD. Statistical significance was determined by *t* test. Statistical significance is considered for $P < 0.05$.

## Results

Genomic DNA and cDNA from the adenoma were analyzed for mutations in the genes encoding Gsα, sst2, and sst5. By analyzing the hot spots of the Gsα gene, a heterozygote point mutation at codon 201 (CGT > TGT/Arg201Cys), known to constitutively activate adenylyl cyclase (20), was identified. By fully sequencing the sst2 gene, no nucleotide substitution in the coding or bordering regions was found, whereas two heterozygote C-to-T substitutions were detected in the sst5 gene. One replaced Pro with Ser (CCC > TCC) at codon 109 in the first extracellular loop, and the other replaced Arg with Trp (CGG > TGG) at codon 240 in the third intracellular loop of the receptor (Fig. 1). Direct sequencing of PCR-amplified cDNA from the tumor revealed WT and mutant bases at about equal intensities. These two substitutions were germ line because they were also identified in the peripheral DNA of the patient. By direct sequencing PCR products generated from 50 unaffected control subjects, Pro109Ser substitution resulted to be an unreported polymorphism because it was found in 3 of 100 normal alleles, and Arg240Trp resulted to be a mutation because it was detected only in the patient. Subcloning of the patient genomic DNA showed that the polymorphism was present in cells expressing sst5 with the Arg240Trp mutation and not in cells expressing the WT receptor, indicating that one sst5 allele had both the mutation and the polymorphism. Owing to the presence of a germ line mutation, we planned to screen the patient's family. Unfortunately, she was the only child of two unrelated parents who died of cardiovascular diseases. Genomic DNA from peripheral leukocytes of her only daughter, a healthy 25-yr-old woman, showed no substitutions in the sst5 gene.

The functional effects of the Arg240Trp mutation were investigated in CHOK1 cells stably expressing WT or mutant sst5. Analysis of binding data indicated the presence of a single class of high affinity-binding sites with no difference between WT or mutant cells in maximum binding capacity (Bmax:125 ± 15 fmol/mg in WT and 120 ± 10 in mutant cells) and estimated affinity constant ($K_d$: 0.26 ± 0.02 nM in WT and 0.28 ± 0.02 in mutant cells). Somatostatin-28 induced a similar inhibition (78 ± 3%) of forskolin-stimulated cAMP accumulation in all clones tested. However, the potency of the peptide was different since the maximal effect occurred at 0.1 nM in WT and at 1 nM in mutant cells (Fig. 2). Cells expressing WT or mutant receptors showed a different growth rate, because the proliferation of mutant cells exceeded that of WT by 50% after 72-h incubation (Fig. 3A). Incubation with the somatostatin analog octreotide (1 nM for 24 h) caused a significant reduction of FCS-induced proliferation of WT cells (25 ± 2% inhibition, $P < 0.001$), that was completely prevented by pretreatment with pertussis toxin (100 ng/ml for 24 h). Conversely, in mutant cells octreotide did not cause any reduction of growth rate (Fig. 3B). Moreover, in cells with WT sst5, 1 nM somatostatin-28 induced a significant inhibi-

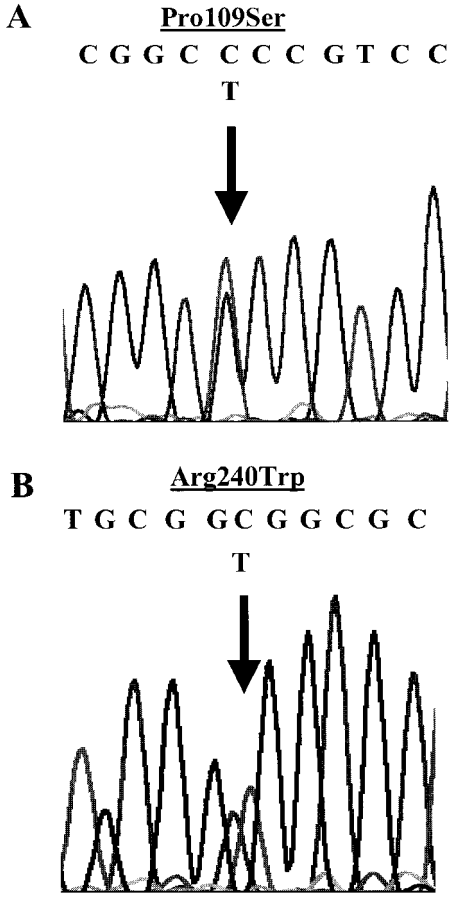


FIG. 1. Sequence analysis of the sst5 gene revealed two heterozygote C-to-T substitutions. A, One at codon 109 (Pro109Ser) in the first extracellular loop; B, the other at codon 240 (Arg240Trp) in the third intracellular loop of the receptor. Pro109Ser substitution resulted to be a polymorphism found in 3 of 100 normal alleles, and Arg240Trp resulted in a mutation because it was detected only in the patient.

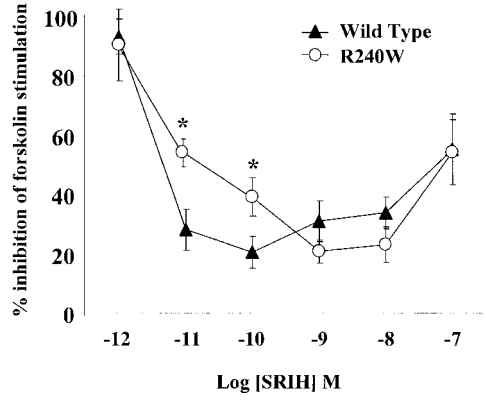


FIG. 2. Effect of native somatostatin (Somatostatin-28; SRIH) on forskolin-stimulated cAMP accumulation in WT (WTsst5/15, WTsst5/20, and WTsst5/25) and mutant (R240Wsst5/6, R240Wsst5/26, and R240Wsst5/27) clones. The maximal inhibition was observed at 0.1 nM in WT and at 1 nM in mutant cells. Data are the mean ± SD of cAMP determinations carried out in triplicate for each clone tested. * $P < 0.05$

tion of FCS-stimulated MAPK activities, whereas in mutant cells the peptide did not reduce but even increased this activity by 2- to 3-fold. These data were confirmed by detecting the levels of phosphorylated p44 and p42MAPK by Western blotting (Fig. 4).

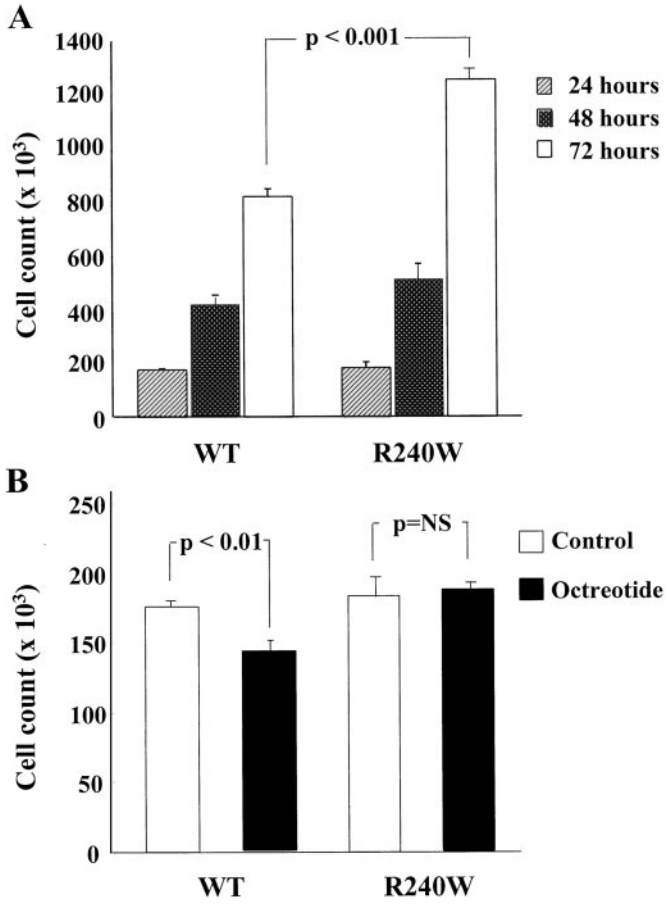


FIG. 3. A, Different proliferation rate of WT (WTsst5/20 and WTsst5/25) and mutant (R240Wsst5/26 and R240Wsst5/27) clones. Cells were cultured in αMEM containing 10% FCS and plated in 35-mm dishes. After 24-, 48-, and 72-h incubation, the number of WT or mutant cells was evaluated using a Coulter counter. B, Effect of 1-nM octreotide on FCS-stimulated cell proliferation in WT and mutant clones. After 24 h of incubation, cells were counted using a Coulter counter. Data are the mean ± SD of cell number determinations carried out in triplicate for each clone tested

Finally we investigated the expression of sst2 and sst5 in the tumor tissue. The levels of sst2 mRNA were markedly lower than those observed in responsive GH-secreting adenomas (sst2/GAPDH ratio, 0.14 *vs.* 1.02 ± 0.30), and no difference was observed in sst5 mRNA (sst5/GADPH ratio, 2.45 *vs.* 2.53 ± 1.15). These data were similar to those observed in a series of tumors removed from resistant patients (personal observations).

## Discussion

In this study we describe the first mutation of a receptor of the sst family in one acromegalic patient resistant to treatment with the somatostatin analog octreotide. This mutation, replacing Arg 240 with Trp in the sst5 gene, is located in the 22-residue region of Lys224 to Arg245 within the third cytoplasmic loop of the receptor. This region represents the cytoplasmic extension of the α-elix 6 and is a consensus sequence identifying a potential site for coupling with G proteins (12, 13). In human and rat sst3–5, Arg is present at codon 240, whereas in sst1 and sst2, it is replaced by Lys, another hydrophilic and positively charged amino acid. The replacement of Arg240 with Trp, an aromatic and highly hydrophobic amino acid, is predicted to alter critical electrostatic interhelical interactions, leading to altered G protein coupling. Indeed, several single amino acid substitutions in other G protein-coupled receptors resulting in altered signaling have been localized in this motif of the third cytoplasmic loop (30, 31).

The expression of the mutant receptor in CHOK1 cells resulted in profound alterations of somatostatin signaling. Although both the WT and mutant receptors appeared to be effective in inhibiting cAMP accumulation, the mutant sst5 required 10-fold higher somatostatin concentrations than the WT to induce the same inhibitory effect. As already reported (12, 13), in WT cells somatostatin analog caused a reduction of cell growth by interacting with a pertussis toxin-sensitive G protein coupling. The antiproliferative effect mediated by sst5, which has been reported to be independent from cAMP inhibition and phosphatase stimulation, was associated with an inhibition of MAPK activity (12, 13, 18). The expression of mutant receptor totally abrogated the inhibitory action of somatostatin on cell growth and MAPK activity and was associated with a higher rate of cell proliferation. Indeed, the mutant receptor stimulated the serum stimulated MAPK

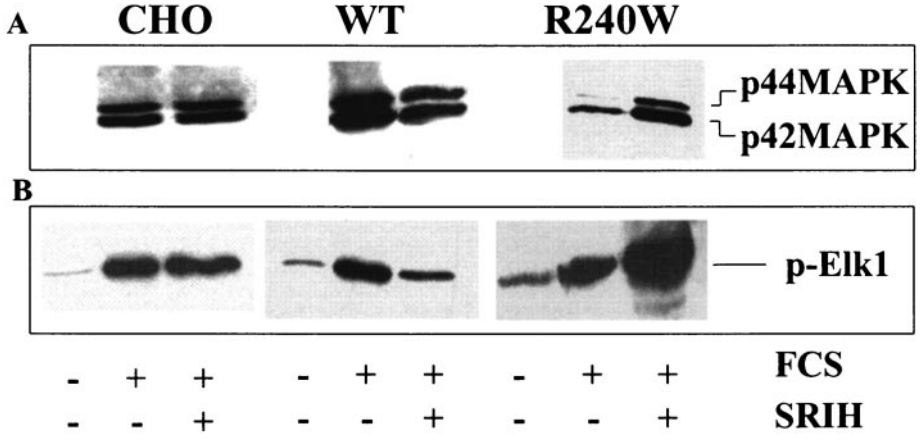


FIG. 4. A, Representative immunoblotting performed with antibodies raised against p42 and p44 MAPK. Each lane was loaded with 20 μg cell lysate obtained from WT (WTsst5/25) and mutant cells (R240Wsst5/27). B, Effect of somatostatin (SRIH, 1 nM) on FCS-stimulated MAPK activity in mutant (R240W) and WT sst5 clones. Kinase activity was performed after immunoprecipitation and p-ELK1 was quantitated by an imaging densitometer. Similar results were obtained in WT (WTsst5/15 and WTsst5/20) and mutant (R240Wsst5/6 and R240Wsst5/26) clones.

activity, suggesting that replacement of Arg 240 by Trp confers to the receptor the ability to couple with G proteins different from those activated by the WT sst5 and able to signal to the MAPK cascade via Gα (such as Gq) or βγ subunits (32, 33). To the best of our knowledge, this is the first example of how receptor mutations may alter G protein coupling, thus changing an inhibitory signal into a stimulatory input.

The relative role of sst2 and sst5 in the control of GH secretion in acromegalic patients is still unclear, and both receptors are probably required for hormonal control because the activation of sst2 and sst5 results in a synergistic effect on GH release (26, 34). This view is also supported by the observation that these receptors may form heterodimers with enhanced functionality (35) As far as the expression of receptor subtypes in GH-secreting adenomas is concerned, it is well documented that sst5 is the most abundantly expressed receptor, sst5 mRNA levels being 10-fold higher than sst2 mRNA (26). This ratio was even higher in the present adenoma, which showed a selective loss of sst2 mRNA, as frequently occurs in poorly responsive tumors (24–26). Therefore, although the phenotype resulting from the expression of mutant sst5 has been characterized in cells without secretory properties, such as CHOK1, we suggest that the mutation of sst5 gene together with the low expression of sst2, may be responsible for the resistance to octreotide observed in this patient. The presence of a germ-line mutation is consistent with the phenotype of the patient because the expression of this receptor is restricted to specific cell types. Indeed, the patient was resistant to somatostatin at the two target organs in which sst5 is mainly expressed and involved in hormonal control, that is somatotrophs and pancreatic β cells (36). In conclusion, in this patient we identified two molecular abnormalities occurring on different loci; the first is the germ-line mutation of sst5 that abrogates the antiproliferative action of somatostatin and activates mitogenic pathways, and the second is the somatic mutation of Gsα that constitutively activates cAMP formation, this effect being poorly counteracted by the mutant receptor.

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