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Absence of Constitutively Activating Mutations in the GHRH Receptor in GH-Producing Pituitary Tumors

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The molecular events leading to the development of GH-producing pituitary tumors remain largely unknown. We hypothesized that activating mutations of the GHRH receptor might occur in a subset of GH-producing pituitary tumors. Genomic DNA samples from 54 GH-producing pituitary tumor tissues were screened for mutations of the GHRH receptor. Eleven homozygous or heterozygous nucleotide substitutions [169G > A (A57T), 338C > T (P113L), 363G > T (E121D), 409C > T (H137Y), 547G > A (D183N), 673G > A (V225I), 749G > A (W250X), 760G > A (V254M), 785G > A (S262N), 880G > A (G294R), 1268G > A (C423Y)] were found in 12 patients (22.2%). The 169G > A substitution (A57T) appears to be a polymorphism (4 patients, 7.4%). E121D and V225I were each found in 2 patients. In 1 patient with the V225I sequence, the substitution was not found in genomic DNA from peripheral leukocytes, suggesting a somatic mutation. A patient with a heterozygous W250X mutation was homozygous for the C423Y substitution.

These variant GHRH receptors were studied in transfected TSA-201 cells to evaluate the functional consequences of the amino acid changes. None of the GHRH receptor variants was associated with basal elevation of intracellular cAMP. GHRH induced variable cAMP responses. With the W250X and G294R variants, there was no cAMP stimulation by GHRH, indicating that the mutations are inactivating. Expression of the W250X GHRH receptor on the cell membrane was severely decreased and GHRH binding to the G294R GHRH receptor was impaired.

Although GHRH receptor variants are common in GH-producing pituitary adenomas, constitutively activating mutations, as a mechanism for GH-producing pituitary tumors appear to be rare. (*J Clin Endocrinol Metab* 86: 3989–3995, 2001)

HYPOTHALAMIC GHRH stimulates the synthesis and secretion of GH from the anterior pituitary. GHRH binds to its stimulatory G protein-coupled receptor at the cell membrane, activating adenylyl cyclase and increasing intracellular cAMP levels. Somatic mutations that inhibit GTPase activity of the G protein α chain (Gsα) are a well established molecular cause of GH-producing pituitary tumors. These mutations have been identified in 30–40% of GH-producing pituitary tumors (1, 2). Activating mutations of G protein-coupled-coupled receptors (GPCRs) have also been found in many endocrine diseases, such as hyperfunctioning thyroid adenomas (TSH receptor) (3), familial male-limited precocious puberty (LH receptor) (4), and Jansen's metaphyseal chondrodysplasia (PTHrP receptor) (5). These mutations confer constitutive activation to the receptors and result in cellular proliferation and increased hormone synthesis and secretion. Based on these observations, we hypothesized that activating mutation of GHRH receptor might occur in a subset of GH-producing pituitary adenomas. In this study, we analyzed the GHRH receptor gene in 54 GH-producing pituitary tumors. Several GHRH receptor variants were identified and their functional characteristics are described.

Materials and Methods

Subjects and isolation of DNA

Fifty-four patients with GH-producing pituitary tumors were included in this study. The diagnosis of a GH-secreting tumor was established by measurement of GH and IGF-1 and confirmed in all cases by positive immunohistochemical staining of tumor tissue for GH. The study protocol was approved by the hospital committees of Yonsei Medical Center and Northwestern University Medical School, and written consent was obtained from all patients.

Genomic DNA was extracted from paraffin-embedded pituitary tumor specimens. Five to seven adjacent 5-μm sections were cut from each paraffin block and mounted on slides. The middle section was stained with hematoxylin-eosin and served to distinguish tumor tissue from normal surrounding tissue. DNA was isolated from tumors sections as previously described (6).

Sequence analysis

Pairs of primers flanking each exon of the GHRH receptor (Table 1) were designed based on recently identified sequence information. A 127-kb fragment from the human p14-p15 region of chromosome 7 containing the complete GHRH receptor with its introns and promoter region has been sequenced as part of the Human Genome Project (7). PCR primers were designed with additional 5′ sequences for universal primers (−21 M13 or M13 reverse) to facilitate sequencing. The PCR (50 μl) included 300 ng DNA, 10 pmol of each primer, 50 μM deoxynucleoside triphosphate (deoxy-NTPs), 5 mM $MgCl_2$ in PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100) containing 2.5 U *Taq* DNA polymerase (Promega Corp., Madison, WI). Cycle conditions were: 2 min hot start at 96 C, followed by 35 cycles of 1 min at 94 C, 45 sec at 58 C, and 45 sec at 72 C, followed by extension at 72 C for 15 min. The PCR products were resolved on 1.5% agarose gels and purified as described previously (8). Direct DNA sequencing was performed using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction kits with AmpliTaq DNA Polymerase FS (Perkin-Elmer Corp., Foster City, CA) and the ABI PRISM 377 DNA automated Sequencer (Perkin-Elmer Corp.).

Direct DNA sequencing was used to identify mutations in Gsα exon 8 (codon 201) and exon 9 (codon 227). Amplified products were purified and sequenced as described above.

Abbreviations: GPCRs, G protein-coupled-coupled receptors; Gsα, G protein α chain; IL, intracytoplasmic loop; PACAP, pituitary adenylate cyclase-activating polypeptide; TM, transmembrane.

TABLE 1. Primers used to amplify the 13 exons of the GHRH receptor

Exon	Sense	Antisense
1	GATAGCCAAGGCTTACTGAG	AGTTGGCCCTGTGGCTCC
2	AGAGCCAGAAAGACACCC	GAGGATATAAAGGAATCAGAGA
3	CCTGCACCTGGGCTGAGT	GCCACTTCCAGATGAAAGCA
4	CCTGGGGAGAGGGAAGGA	CCCATACAAATGTCCATGGC
5	TTCCTCCAAAGGCCCAGAAG	AGCAGACTTGACCCTCTGG
6	CTCATTTCTCCCATTACCCC	CCTGCCCAGCCCCTTCAC
7	CTGGGATGGGGCTCCAGT	TGGGCCTTTCCAGCCCTTT
8	CAGAGTCAAGGATGCAGACT	AGCCCATGGCCTGACTGT
9	GTCACCACAGTGAAGGGG	AGTAGTTCCAGGGAAGTTGA
10	TGTCTTCCACCTTCCTAT	CTTATTGGATGAGACAGA
11	GGAGCCTAGGATTTGTCTT	AGAAAGAGTGCCTGTGGG
12	AAAGGTAGCAGAAAGACGGTG	GGAGGGGCCTTAGGTCTG
13	GGTTGAGTAGCAAAGCCACA	GGCAGACCCGTGGTAGCT

Construction of GHRHR variants and generation of HA epitope-tagged receptor constructs

The wild-type GHRH receptor cDNA (1617 bp) was inserted into the pcDNA-3 vector (Invitrogen, Carlsbad, CA) downstream of the T7 RNA polymerase promoter and flanked by *Bam*HI and *Xho*I sites at the 5′ and 3′ ends, respectively. Using site-directed mutagenesis, GHRH receptor variants were generated. The wild-type and variant GHRH receptors were subcloned into the pSVL plasmid (Pharmacia Biotech, Piscataway, NJ) at *Bam*HI and *Xho*I sites (created by oligonucleotide annealing).

Fusion constructs were created by incorporating an N-terminal influenza virus HA epitope (YPYDVPDYA) in frame with the GHRH receptor (wild-type and variants) in the pSVL vector. All the final constructs were verified by DNA sequencing.

Immunocytochemical analysis of GHRH receptor expression

TSA-201 (hereafter referred to TSA) cells, derived from human embryonic kidney 293 cells were maintained in DMEM supplemented with 10% FBS and transfected using the calcium phosphate method. Forty-eight hours after transfection of the GHRH receptor or HA-epitope tagged GHRH receptors, TSA cells were collected, washed twice with PBS, mounted on glass slides, and air-dried for 10 min. Slides were fixed for 5 min with 4% paraformaldehyde in sodium phosphate (pH 7.2) and treated with 3% hydrogen peroxide in PBS for 10 min. After preincubation with serum blocking solution (Zymed Laboratories, Inc., San Francisco, CA), specimens were incubated with rat monoclonal anti-HA high affinity antibody (2.5 μg/ml, Roche Molecular Biochemicals, Indianapolis, IN) for 2 h at room temperature. After washing with TBS/0.025% Tween, slides were incubated with antirat Ig-biotin, F(ab′)2 fragment (5 μg/ml, Roche Molecular Biochemicals) for 20 min at room temperature. Streptavidin-peroxidase (Zymed Laboratories, Inc.) was used with the 3-amino-9-ethylcarbazole chromogen (DAKO Corp., Carpinteria, CA). For permeabilized cells transfected with the HA-tagged W250X GHRH receptor, slides were fixed in ice cold methanol (5 min) and acetone (5 min) and boiled in 0.02% NP40/sodium citrate buffer (pH 6.0) for 10 min using a microwave oven. Slides were then treated with 3% hydrogen peroxide in methanol for 10 min. After preincubation with serum blocking solution, the subsequent steps were performed as described above.

Immunofluorescent studies were also performed in TSA cells transfected with GHRH receptors. A rabbit antihuman GHRH-receptor antibody (1: 1000, provided by Dr. Bruce Gaylinn, University of Virginia, Charlottesville, Virginia) was used to detect the GHRH receptor. After incubation for 1 h with the primary antibody, staining was performed using biotinylated secondary antibodies (ABC kit, Vector Laboratories, Inc., Burlingame, CA) and streptavidin-FITC (1:100, Vector Laboratories, Inc.). Cell images were analyzed using a Carl Zeiss microscope (Axioskop, Carl Zeiss Inc., Oberkochen, Germany).

RNA isolation and RT-PCR for the GHRH receptor

To verify expression of the GHRH receptor constructs, total RNA was isolated from transfected TSA cells using TRIZOL reagent (Life Technologies, Inc., Gaithersburg, MD) as described by the manufacturer. Twenty micrograms of RNA was treated with DNase-I for 30 min at room temperature. Random hexamers were used to synthesize single-strand cDNA using 10 μg of DNase-I treated RNA. A portion (1/40) of the cDNA solution was used for amplification of the human GHRH receptor or GAPDH.

Cycle conditions were: 2 min hot start at 96 C, followed by 30 cycles of 1 min at 94 C, 45 sec at 55 C, followed by 1 min at 72 C, and extension at 72 C for 15 min. An aliquot (20%) of each PCR was resolved by electrophoresis on 1.75% agarose gels and DNA products were visualized with ethidium bromide.

Measurement of ligand binding

TSA cells grown to 50% confluency in DMEM/F12 on 6-well plates were transfected by calcium phosphate precipitation with 1 μg of DNA per well. After 36 h, cells were rinsed with 1.0 ml of binding buffer: HEPES 50 mM, sucrose 100 mM, $CaCl2 \cdot 6H_2O$ 5 mM, ovalbumin 0.1% (wt/vol). ^{125}I -labeled human GHRH 1–44 amide (50,000 dpm) (Amersham Pharmacia Biotech, Piscataway, NJ) was added to each well along with 10^{-12} or 10^{-8} M hGHRH 1–44 amide (Sigma, St. Louis, MO). Incubation was continued at room temperature for 3 h and the cells were rinsed twice with 1.0 ml of binding buffer followed by the addition of 0.5 ml of 1.0 N NaOH. After 1 h, the wells were scraped, and the contents transferred to a glass tube. The wells were washed two additional times with 0.25 ml 1.0 N NaOH, and the solution was transferred to the collecting tubes. Radioactivity was measured in a γ counter (United Technologies Packard, Downers Grove, IL). Data are expressed as the percent of counts bound by the wild-type GHRH receptor.

Measurement of intracellular cAMP levels

Triplicate wells of TSA cells, grown to 50% confluency, were transfected with 0.1 μg or 1.0 μg GHRH receptor plasmid DNA per well using the calcium phosphate method. Transfected cells were maintained in DMEM/F12 with 5% dialyzed FBS (10 kDa molecular cut-off, Sigma, St. Louis, MO). Forty-eight hours after transfection, the cells were treated with 0.1 mM isobutylmethylxanthine for 20 min at 37 C. hGHRH 1–44 amide (1 nM) was added in fresh 37 C media, and the incubation was continued for another 30 min at 37 C. The medium was removed and 0.5 ml cold 0.1 M HCl was added to each well. Cell lysates were centrifuged for 10 min at 4 C to remove protein, and the supernatants were neutralized with an equal volume of 150 mM Tris-HCl (pH 8.0) containing 4 mM EDTA. cAMP levels were measured with an RIA Kit (Biomedical Technologies Inc., Stoughton, MA) according to the manufacturer's instructions.

Results

Sequence analysis of GHRH receptor and Gsα

Based on the hypothesis that activating mutations of GHRH receptor might occur independent of Gsα mutations, tumors were first screened for activating mutation of the Gsα subunits. Activating Gsα mutations are located in exons 8 or

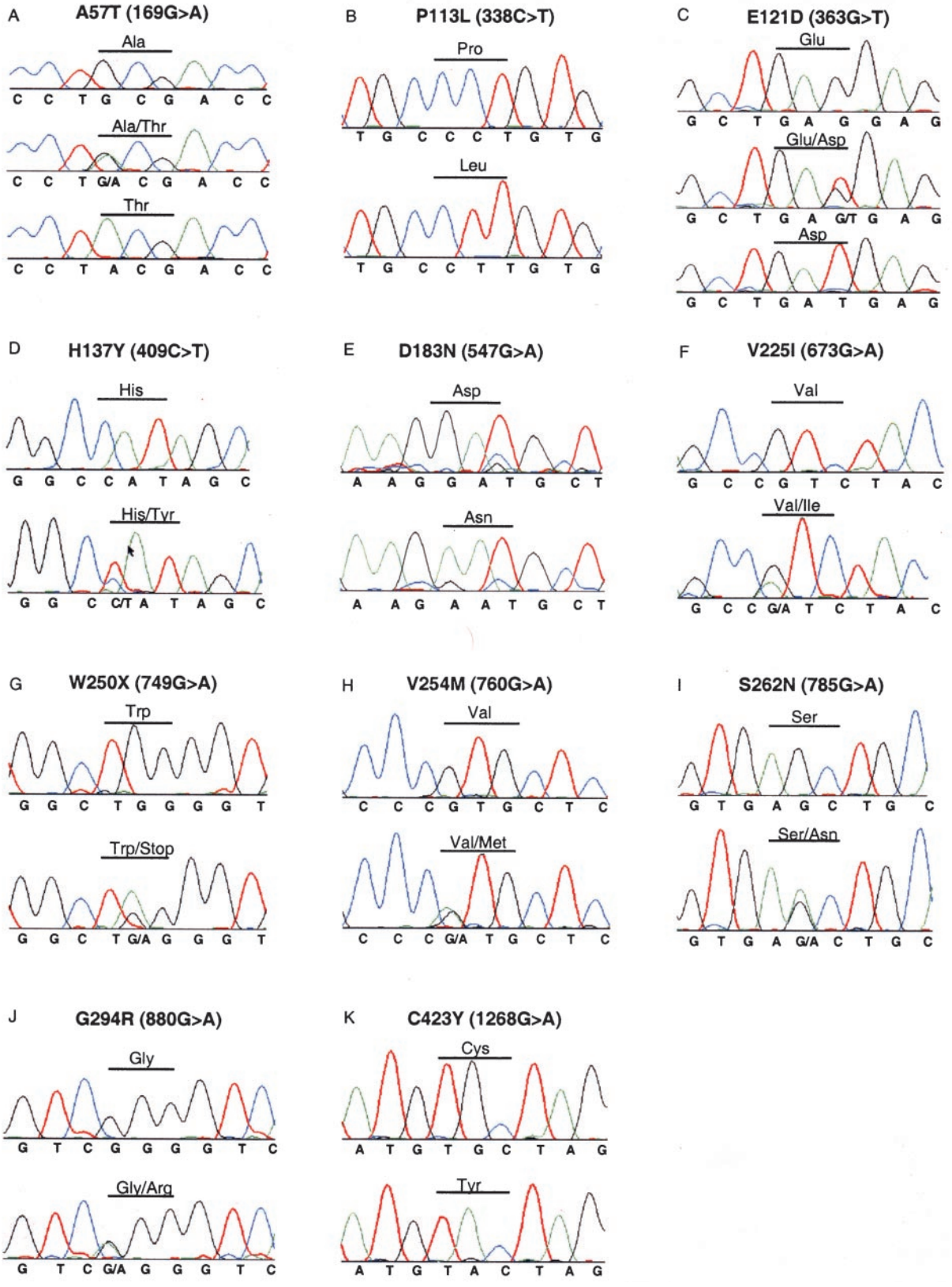

FIG. 1. DNA sequence analysis of the GHRH receptor. Heterozygous or homozygous states of A57T (A), P113L (B), E121D (C), H137Y (D), D183N (E), V225I (F), 250X (G), V254M (H), S262N (I), G294R (J), and C423Y (K). *Upper panel* of each figure is the wild-type GHRH receptor.

9. Therefore, we screened exons 8 and 9 of Gsα and found 15 (27.8%) mutations in 54 acromegalic tumors. Thirteen mutations were located in codon 201 (twelve R201C and one R201H) and two in codon 227 (Q227L), one of which was homozygous.

The nucleotide sequence of the entire coding region of the GHRH receptor gene was analyzed in all 54 tumors. Eleven nucleotide substitutions (169G > A, 338C > T, 363G > T, 409C > T, 547G > A, 673G > A, 749G > A, 760G > A, 785G > A, 880G > A, 1268G > A) were identified in 12 patients (22.2%) of 54 patients (Fig. 1). The resulting amino acid changes are A57T, P113L, E121D, H137Y, D183N, V225I, W250X, V254M, S262N, G294R, and C423Y, respectively. A schematic diagram of the variant GHRH receptors is shown in Fig. 2. Four patients have homozygous variants (A57T and E121D, P113L, D183N, and C423Y). The A57T form of the GHRH receptor was found in 4 patients (7.4%), one of whom also had a Gsα mutation. To determine whether these variations were polymorphisms, we screened 40 genomic DNA samples from peripheral leukocytes of normal persons, 3 of whom (7.5%) were found to have the A57T GHRH receptor. Based on this result, the 169G > A (A57T) substitution is a polymorphism. The E121D substitution was found in two patients who had A57T. One of these individuals was homozygous for both variants. The V225I substitution was also found in two patients, one of whom had A57T. A patient with the W250X termination mutation was also homozygous for C423Y. We obtained peripheral leukocyte DNA from one patient whose tumor had the V225I GHRH receptor. Sequence analysis showed the wild-type genotype of the receptor in peripheral blood, suggesting that the V225I substitution is a somatic mutation.

Expression of GHRH receptors

Immunocytochemistry of aminoterminally HA-tagged GHRH receptors was performed using a monoclonal antibody directed against the HA epitope to assess the membrane expression of variant GHRH receptors. Indirect immunofluorescence was also performed using a rabbit polyclonal antibody directed against the N terminus of the GHRH receptor. The two detection methods gave similar results (data not shown). The wild-type and variant GHRH receptors were expressed on the cell surface (Fig. 3, A and B). All of the variant GHRH receptors showed a similar level of expression with the exception of W250X, which was minimally (2% of normal) expressed (Fig. 3, A-c). Immunocytochemistry of permeabilized cells (data not shown) revealed that the expression level of W250X was similar to that of nonpermeabilized cells. RT-PCR analyses confirmed that the mRNA expression of W250X was similar to that of the wild-type GHRH receptor (Fig. 4). These findings suggest that the W250X protein is unstable and may be degraded in the cytoplasm.

Receptor binding

Ligand competition studies were carried out to assess GHRH binding to GHRH receptor variants. With the excep-

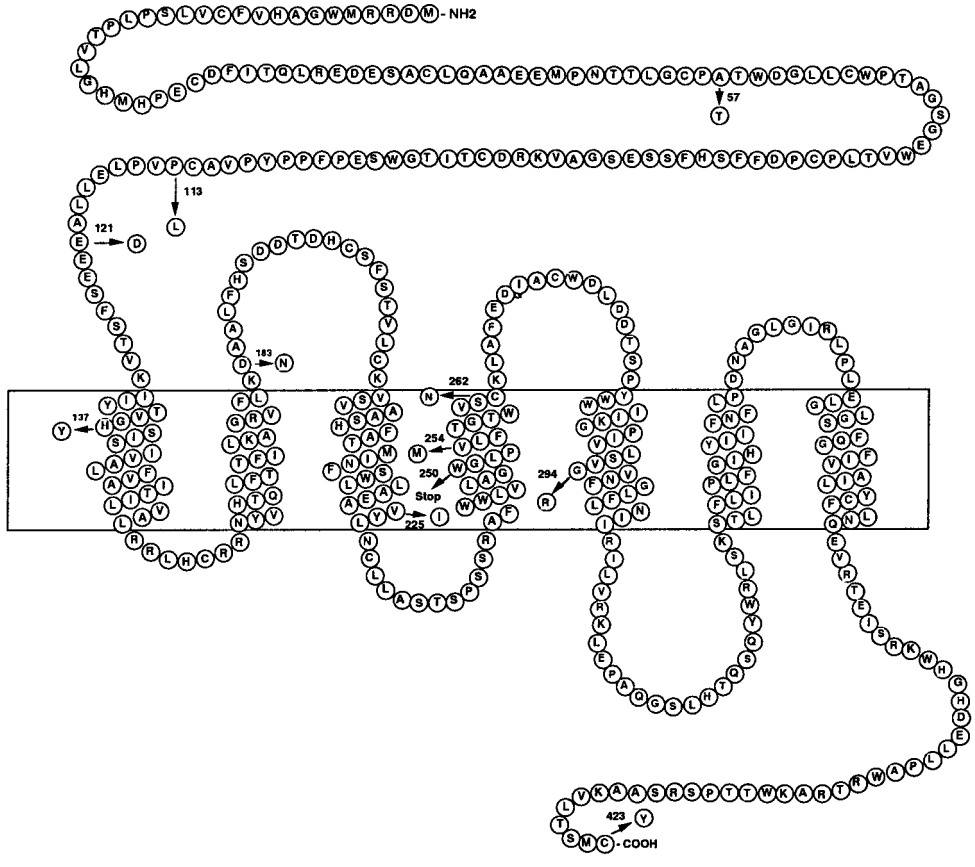

FIG. 2. Schematic presentation of variant GHRH receptors.

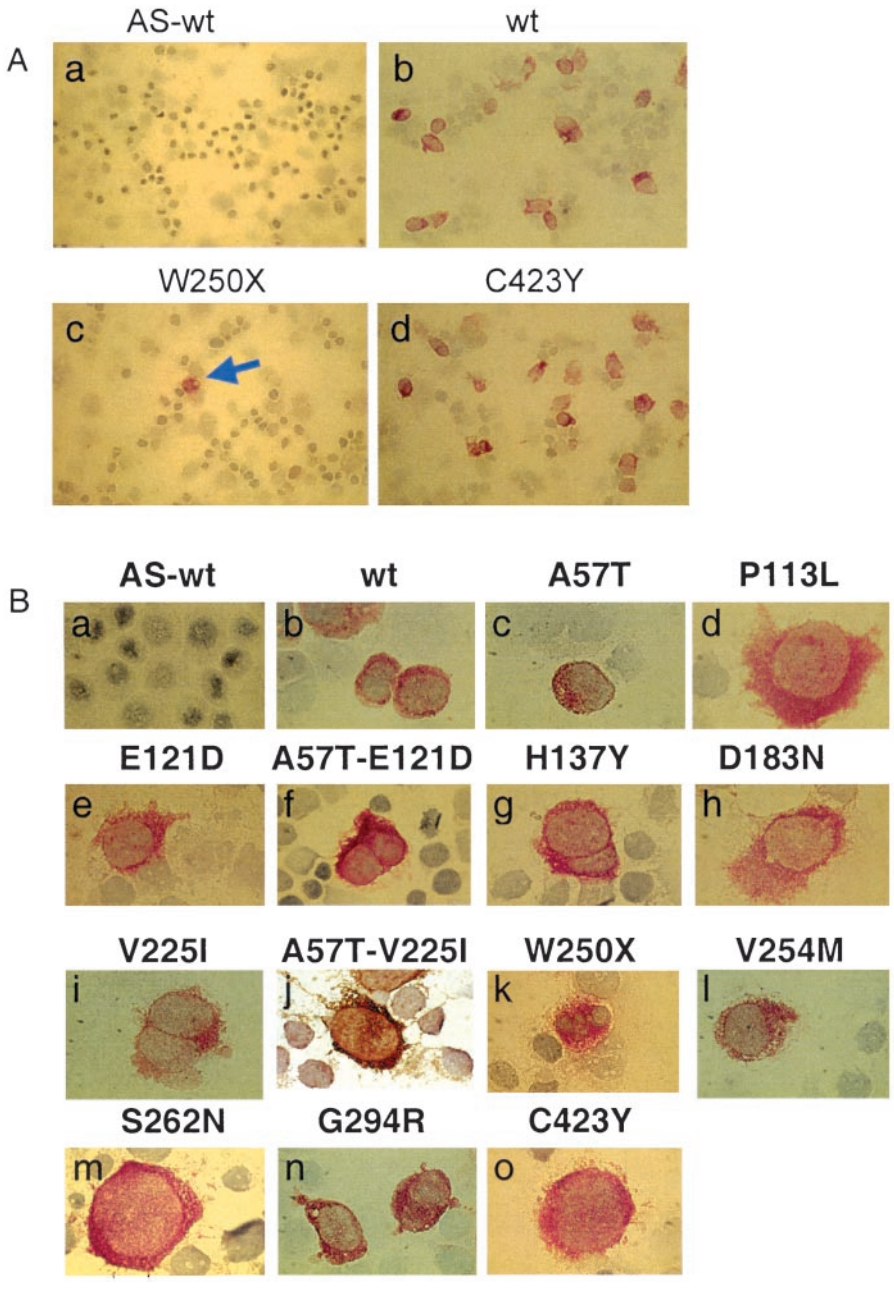

FIG. 3. Expression of GHRH receptors in TSA cells. TSA cells were transfected with expression vectors encoding HA-tagged wild-type and variant GHRH receptors. Nonpermeabilized cells were incubated with rat monoclonal anti-HA high affinity antibody. A, Quantitation of GHRH receptor expression in TSA cells transfected with antisense-wild-type (a), wild-type (b), W250X (c), or C423Y (d) GHRH receptors. B, Immunocytochemical localization of the wild-type and variant GHRH receptors. Wt, the wild-type GHRH receptor. AS-wt, antisense orientation of the wild-type GHRH receptor.

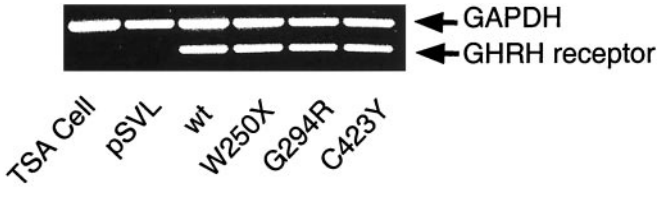

FIG. 4. RT-PCR analysis of GHRH receptor expression in TSA cells. Total RNA was isolated from transfected cells. After RT, the first-strand cDNAs were used as templates to amplify fragments of the human GHRH receptor and GAPDH by PCR. An aliquot (20%) of each PCR was resolved by electrophoresis on 1.75% agarose gels and DNA products were visualized with ethidium bromide.

tion of the G294R, all receptor variants bound GHRH specifically (Fig. 5). Maximum binding (compared with wild-type GHRH receptor) varied from 107% (A57T) to 44% (W250X), and unlabeled ligand competed with radiolabeled GHRH.

Functional analysis of GHRH receptor variants

The wild-type and variant GHRH receptors were expressed in TSA cells to assess their activities in the absence and presence of GHRH. Basal and GHRH-induced activities were assessed by measuring cAMP, the major second messenger for the GHRH receptor. The antisense orientation of wild-type receptor was used as a negative control. To investigate basal cAMP production, two different doses (0.1 μg and 1.0 μg/well) of transfected receptor were analyzed. The basal levels of intracellular cAMP were similar for both doses of wild-type and variant GHRH receptors, and not different from mock transfected cells (data not shown). The addition

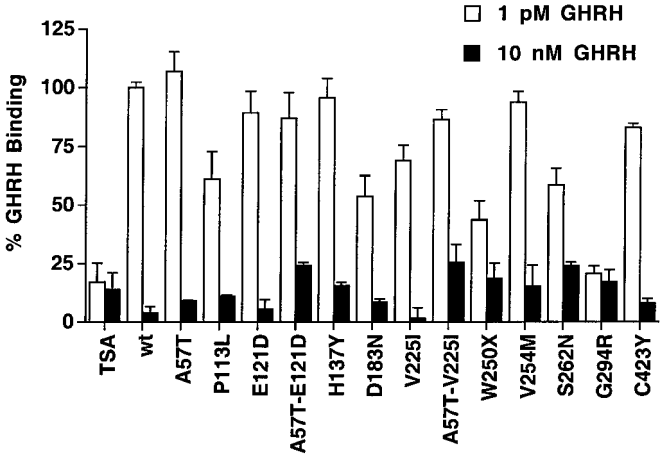

FIG. 5. Binding of GHRH to GHRH receptors in TSA cells transfected with GHRH receptors. ^{125}I-labeled, and two concentrations of unlabeled, human GHRH 1–44 amide were added to plated cells. Results are expressed as a percentage of the maximum specific binding of the wild-type GHRH receptor.

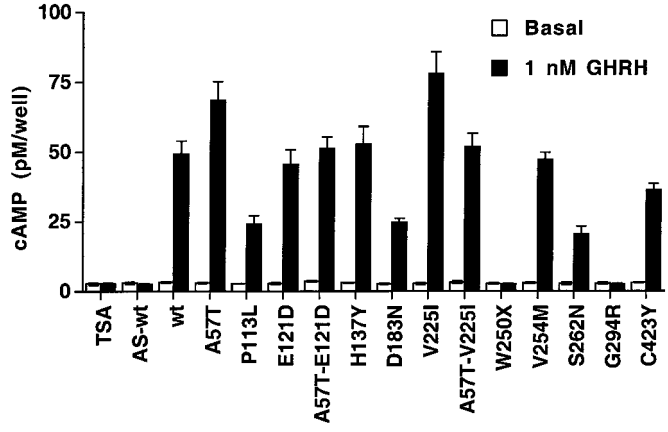

FIG. 6. Basal and GHRH-stimulated cAMP accumulation in TSA cells transfected with wild-type (wt) and variant GHRH receptors. Triplicate wells were transfected with 1.0 μg of expression vectors. Transfected cells were incubated with 1 nM GHRH for 30 min, and intracellular cAMP levels were measured.

of GHRH (1 nM) induced variable degrees of cAMP elevation. No cAMP elevation was detected with the W250X and G294R receptor variants (Fig. 6), indicating that these mutations impair signal transduction by GHRH.

Discussion

Pituitary tumors are known to be monoclonal in origin (9), suggesting that somatic mutations in progenitor cells are initiating events in tumorigenesis. In GH-producing pituitary adenomas, LOH of chromosome 11q13 (10, 11), and activating mutations of Gsα (1, 2, 12) have been identified as pathogenetic mechanisms. The activating mutations of Gsα lead to constitutive activation of adenylyl cyclase with a consequent high rate of cAMP production. Increased cAMP levels activate protein kinase A, which in turn phosphorylatesthe transcription factor CREB. Because this signaling pathway is activated by GHRH, we hypothesized that activating mutations of the GHRH receptor might provide a potential mechanism for initiating or enhancing the growth of GH-producing pituitary tumors. Our efforts were initially focused on possible mutations in the third intracytoplasmic loop (IL) and the sixth transmembrane (TM) domain of the GHRH receptor given the high frequency of activating mutations in these regions in other GPCRs. However, no mutations were found in these regions. Recently, the entire genomic sequence of the human GHRH receptor has been published (7), leading us to assess the entire coding sequence of the GHRH receptor. In 54 acromegalic tumor samples, we identified 11 different nucleotide substitutions. No constitutive activation was found as a result of these alterations, suggesting that activating mutations of the GHRH are probably not a common cause of GH-producing pituitary adenomas.

Recently, two activating mutations (H223R and T410P) in PTH/PTHrP receptor were identified in a patient with Jansen's metaphyseal chondrodysplasia (5, 13). The wild-type amino acids at these sites are highly conserved among same GPCR family B members. H223R and T410P are located at the junction between IL1 and TM2, and IL3 and TM6, respectively. Of the amino acids in variant GHRH receptors in this study, only two, D183N and W250X, are conserved in other GPCR family B members (14, 15). D183N is located at the junction between TM2 and the first extracellular loop (EL1), which is close to the location of H223R PTH/PTHrP receptor. However, the D183N GHRH receptor diminished the cAMP response (40–50% of wild-type) to GHRH, without evidence of basal constitutive activity.

Several lines of evidence suggest that the IL domains and the C-terminal tail of the GPCRs are involved in G protein coupling. Alternative splicing within IL1 of the calcitonin receptor (16, 17) and IL3 of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (18–20), affects receptor coupling to the adenylyl cyclase and PLC pathways. Recently, two alternatively spliced isoforms of the rat GHRH receptor have been identified (21). The long isoform, which has an additional 41 amino acids in IL3, has reduced receptor signaling through the adenylyl cyclase pathway, although the receptor is expressed on the cell surface and binds GHRH. In the W250X variant, which is truncated downstream of EL2, the binding of GHRH was partially maintained, but the cAMP response to GHRH is absent, suggesting that IL3 and the C-terminal tail are important for G protein coupling to the GHRH receptor.

The expression level of G294R was equivalent to the wild-type GHRH receptor, but the single amino acid change in TM5 abolished the receptor's ability to bind GHRH. In several diseases, single point mutations of GPCRs have been shown to decrease the expression of the receptor on the cell surface as well as its affinity for the ligand (22, 23). The G294R mutation decreased ligand binding without affecting the cell surface localization of the receptor, suggesting that that G294R is an inactivating mutation. These characteristics are similar to the features of the mutant GHRH receptor of the *little* mouse (24, 25).

Interestingly, four patients in this study appeared to be homozygous for GHRH receptor variants. Possibly, these patients are actually hemizygous due to LOH in this region of the genome. LOH has been described in many tumors;

adrenocortical tumors (chromosome 18p11.2: ACTH receptor gene) (26), sporadic parathyroid tumors (chromosome 1) (27), pancreatic islet cell tumors (chromosome 3p14.2–3p21) (28), hepatocellular carcinomas (chromosome 8p23) (29), and colorectal carcinomas (chromosome 3p23) (30). These findings suggest that the deletion of specific regions of a chromosome can result in loss of normal differentiation and clonal expansion of malignant cell clones. LOH could not be formally assessed in this study because normal tissue was not available. However, it might be of interest to investigate LOH at the GHRH receptor locus in association with the tumorigenesis of acromegaly.

The lack of obvious gain or loss of function associated with most of the variants described here suggests that the nucleotide substitutions in these variant GHRH receptors may be polymorphisms. The A57T GHRH receptor was found in 4 cases (7.4%) in acromegalic tumor tissue, but it was also found in genomic DNA of 3 (7.5%) of 40 normal subjects. This is consistent with another report demonstrating that A57T is a polymorphism (31). V225I was not found in genomic DNA from the patient's leukocytes, suggesting that V225I is a somatic mutation in the tumor cell.

The pathogenesis of a large subset of pituitary tumors causing acromegaly is not well understood. In this study, genetic abnormalities of the GHRH receptor were investigated and several variant GHRH receptors were identified in human acromegalic pituitary tumor tissues. None of them was associated with constitutive activation of the cAMP pathway. Although some of these mutations may affect tumorigenesis by other mechanisms, activating mutations of the GHRH receptor are probably not a common cause of human GH-producing pituitary tumors.

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References

1. **Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L** 1989 GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. Nature 340:692–696
2. **Adams EF, Brockmeier S, Friedmann E, Roth M, Buchfelder M, Fahlbusch R** 1993 Clinical and biochemical characteristics of acromegalic patients harboring gsp-positive and gsp-negative pituitary tumors. Neurosurgery 33:198–203; discussion 203
3. **Parma J, Sande JV, Swillens S, Tonacchera M, Dumont J, Vassart G** 1995 Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: identification of additional mutations activating both the cyclic adenosine 2′ 5′-monophosphate and inositol phosphate-Ca^{2+} cascades. Mol Endocrinol 9:725–733
4. **Shenker A, Laue L, Kosugi S, Merendino Jr JJ, Minegishi T, Cutler Jr GB** 1993 A constitutively activating mutation of the lutenizing hormone receptor in familial male precocious puberty. Nature 365:652–654
5. **Schipani E, Langman CB, Parfitt AM, et al.** 1996 Constitutively activated receptors for parathyoid hormone and parathyoid hormone-related peptide in Jansen's metaphyseal chondroplasia. N Engl J Med 335:708–714
6. **Karga H, Lee JK, Vickery Jr AL, Thor A, Gaz RD, Jameson JL** 1991 ras oncogene mutations in benign and malignant thyroid neoplasms. J Clin Endocrinol Metab 73:832–836
7. **Andrew S, Dubbelde C, Ryan E** 1998 The sequence of Homo sapiens PAC clone DJ0877J02 GenBank AC005155
8. **Jackson D, Hayden J, Quirke P** 1991 Extraction of nucleic acid from fresh and archival material. In: M. J. McPherson PQ, Taylor GR, eds. PCR: a practical approach. New York: Oxford University Press; 29–50
9. **Herman-Bonert V, Fagin JA** 1995 Molecular pathogenesis of pituitary tumours. Baillieres Clin Endocrinol Metab 9:203–223
10. **Tanaka C, Kimura T, Yang P, et al.** 1998 Analysis of loss of heterozygosity on chromosome 11 and infrequent inactivation of the MEN1 gene in sporadic pituitary adenomas. J Clin Endocrinol Metab 83:2631–2634
11. **Gadelha MA, Prezant TR, Une KN, et al.** 1999 Loss of heterozygosity on chromosome 11q13 in two families with acromegaly/giantism is independent of mutation of the multiple endocrine neoplasia type gene. J Clin Endocrinol Metab 84:249–256
12. **Spiegel AM** 1996 Mutations in G proteins and G protein-coupled receptors in endocrine disease. J Clin Endocrinol Metab 80:2490–2494
13. **Schipani E, Jensen GS, Pincus J, Nissenson RA, Gardella TJ, Juppner H** 1997 Constitutive activation of the cyclic adenosine 3′,5′-monophosphate signaling pathway by parathyroid hormone (PTH)/PTH-related peptide receptors mutated at the two loci for Jansen's metaphyseal chondrodysplasia. Mol Endocrinol 11:851–858
14. **Gaylinn BD, Harrison JK, Zysk JR, Lyon CE, Lynch KR, Thorner MO** 1993 Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. Mol Endocrinol 7:77–84
15. **Mayo KE** 1992 Molecular cloning and expression of a pituitary specific receptor for growth hormone-releasing hormone. Mol Endocrinol 6:1734–1744
16. **Nussenzveig DR, Thaw CN, Gershengorn MC** 1994 Inhibition of inositol phosphate second messenger formation by intracellular loop one of a human calcitonin receptor. Expression and mutational analysis of synthetic receptor genes. J Biol Chem 269:28123–28129
17. **Moore EE, Kuestner RE, Stroop SD, et al.** 1995 Functionally different isoforms of the human calcitonin receptor result from alternative splicing of the gene transcript. Mol Endocrinol 9:959–968
18. **Spengler D, Waeber C, Pantaloni C, et al.** 1993 Differential signal transduction by five splice variants of the PACAP receptor. Nature 365:170–175
19. **Journot L, Spengler D, Pantaloni C, Dumuis A, Sebben M, Bockaert J** 1994 The PACAP receptor: generation by alternative splicing of functional diversity among G protein-coupled receptors in nerve cells. Semin Cell Biol 5:263–272
20. **Chatterjee TK, Sharma RV, Fisher RA** 1996 Molecular cloning of a novel variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor that stimulates calcium influx by activation of L-type calcium channels. J Biol Chem 271:32226–32
21. **Miller TL, Godfrey PA, Dealmeida VI, Mayo KE** 1999 The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signaling properties. Endocrinology 140:4152–4165
22. **Pan Y, Wilson P, Gitschier J** 1994 The effect of eight V2 vasopressin receptor mutations on stimulation of adenylyl cyclase and binding to vasopressin. J Biol Chem 269:31933–31937
23. **Karaplis AC, He B, Nguyen MTA, et al.** 1998 Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Bloomstrand chondroplasia. Endocrinology 139:5255–5288
24. **Godfrey G, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE** 1993 GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. Nat Genet 4:227–232
25. **Gaylinn BD, Dealmeida VI, Lyons Jr CE, Wu KC, Mayo KE, Thorner MO** 1999 The mutant growth hormone-releasing hormone (GHRH) receptor of the little mouse does not bind GHRH. Endocrinology 140:5066–5074
26. **Latronico AC** 2000 Role of ACTH receptor in adrenocortical tumor formation. Braz J Med Biol Res 33:1249–1252
27. **Dwight T, Twigg S, Delbridge L, et al.** 2000 Loss of heterozygosity in sporadic parathyroid tumours: involvement of chromosome 1 and the MEN1 gene locus in 11q13. Clin Endocrinol (Oxf) 53:85–92
28. **Nikiforova MN, Nikiforov YE, Biddinger P, et al.** 1999 Frequent loss of heterozygosity at chromosome 3p14.2–3p21 in human pancreatic islet cell tumours [see comments]. Clin Endocrinol (Oxf) 51:27–33
29. **Liao C, Zhao M, Song H, Uchida K, Yokoyama KK, Li T** 2000 Identification of the gene for a novel liver-related putative tumor suppressor at a high-frequency loss of heterozygosity region of chromosome 8p23 in human hepatocellular carcinoma. Hepatology 32:721–727
30. **Iniesta P, Massa MJ, Gonzalez-Quevedo R, et al.** 2000 Loss of heterozygosity at 3p23 is correlated with poor survival in patients with colorectal carcinoma. Cancer 89:1220–1227
31. **Adams EF, Symowski H, Buchfelder M, Poyner DR** 2000 A polymorphism in the growth hormone (GH)-releasing hormone (GHRH) receptor gene is associated with elevated response to GHRH by human pituitary somatotrophinomas in vitro. Biochem Biophys Res Commun 275:33–36