Recessive Isolated Growth Hormone Deficiency and Mutations in the Ghrelin Receptor

Jacques Pantel,* Marie Legendre,* Sylvie Nivot, Séverine Morisset, Marie-Pierre Vie-Luton, Yves le Bouc, Jacques Epelbaum, and Serge Amselem

INSERM (J.P., M.L., M.-P.V.-L., S.A.), U933, 75011 Paris, France; Centre Hospitalier Universitaire de Rennes (S.N.), Unité d’Endocrinologie Pédiatrique, 35056 Rennes, France; INSERM (S.M.), U573, 75014 Paris, France; AP-HP (Y.L.B.), Hôpital Armand Trousseau, Service d’Explorations Fonctionnelles Endocriniennes, 75771 Paris, France; and INSERM (J.E.), U549, 75014 Paris, France

Context: Both GH releasing- and orexigenic properties of the gut-to-brain hormone ghrelin are mediated by the GH secretagogue receptor (GHSR). Recently in several patients, a missense mutation (p.A204E) resulting in a complete loss of GHSR constitutive activity has been implicated in short stature with dominant transmission.

Objective: The objective of the study was to describe the phenotype associated with partial isolated GH deficiency of a young patient born to unrelated parents and identify the molecular basis of his disease.

Results: The growth delay (–3.0 SD) was associated with recurrent episodes of abdominal pain, vomiting, ketosis, hypoglycemia, and a low body mass index. GHSR sequencing revealed that the patient was compound heterozygous for two new defects: 1) an early occurring transition predicting a premature stop codon (c.6G>A, p.W2X) inherited from his unaffected father, therefore strongly arguing against haploinsufficiency as a disease mechanism, and 2) a missense mutation (c.709A>T, p.R237W) inherited from his healthy mother, involving an evolutionary invariant residue from the third intracellular loop. In vitro experiments showed that the p.R237W mutation would result in a partial loss of constitutive activity of the receptor, whereas both its ability to respond to ghrelin and its cell surface expression are preserved.

Conclusion: These data, which describe the first case of recessive partial isolated GH deficiency due to GHSR mutations and emphasize the physiological importance of the GHSR in somatic growth, are discussed in light of the dominantly expressed p.A204E mutation. (J Clin Endocrinol Metab 94: 4334–4341, 2009)

A key element of the so-called gut-to-brain axis is ghrelin, a hormone initially depicted as a GH secretagogue (1, 2) but actually endowed with a wide range of pleiotropic functions on metabolic, cardiovascular, gastrointestinal, gonadal, and immune systems as well as sleep and memory (3–8). Both GH-releasing and orexigenic properties of this gut-derived circulating hormone are mediated by the sole receptor known to date, the GH secretagogue receptor (GHSR) (9), a seven-transmembrane G protein-coupled receptor (GPCR) that is highly expressed in the pituitary and the hypothalamus (10) and for which in vitro studies (11) demonstrated a constitutive activity (CA). To assess the physiological importance of the ghrelin axis, several mouse models carrying a targeted disruption of the gene encoding the preproghrelin (ghrI−/−) or its receptor (ghsr−/−) were generated by different groups. However, the resulting normal or near-normal phenotype of these animals led to the conclusion that...
ghrelin does not play a key role in somatic growth or food intake (9, 12, 13) and/or that so far unidentified developmentally compensatory mechanisms might occur, as documented for other orexigenic factors (14, 15).

Recently we reported the first functionally significant GHSR mutation in two unrelated families of the same geographic origin. The patients presented with short stature, with or without isolated GH deficiency (IGHD). Unexpectedly, some of them were shown to be overweight or obese (16). The phenotype segregated in a dominant or semidominant manner with incomplete penetrance and variable expressivity. Functional studies of the corresponding mutated ghrelin receptor revealed that the identified missense mutation (p.A240E), which results in decreased cell surface expression of the receptor, selectively impairs its constitutive activity but preserves its ability to respond to ghrelin.

We now report a patient with partial IGHD, carrying two new GHSR genetic defects and born to two heterozygous parents of normal stature.

### Subjects and Methods

#### Subjects

The propositus is a young boy, born to unrelated parents at 34.5 wk of pregnancy with normal birth height (47 cm) and weight (2860 g). He was first seen at the age of 3 yr for episodes of abdominal pain, vomiting, hypoglycemia, and ketosis, in the context of a postnatal growth delay (3.0 SD below the mean for age) associated with a low body mass index (third percentile), requiring endocrine evaluation. His two siblings and parents have a normal phenotype (Fig. 1) as well as his older half-brother (same mother), who reached a normal adult height. All the other family members are of normal stature, except a female first cousin of the mother who is of very short stature (adult height 138 cm) but has not been investigated. Written informed consents were obtained from all family members participating in this study.

### Ghrelin assay

Total and acylated ghrelin levels were assessed by means of an in-house immunoassay as described elsewhere (16).

### Analysis of the GHSR gene

The two GHSR coding exons and their flanking intronic regions were amplified by PCR from genomic DNA obtained from blood samples; the resulting products were sequenced as previously described (16). Segregation analysis of the two newly identified GHSR mutations was carried out by appropriate digestion of the PCR products spanning each mutation site and generated with primers Pa (5′-TTCTCGCTCTCACCTCCCTCTC-3′) and Pb (5′-AGGTTGAGACCCGGCTCTTCGTGGGCAAGCTT-3′) for the p.W2X mutation (XmnI digestion) and with primers Pc (5′-GTCTTTGTCTGTCAGGGCTTCTCTACATCGTCTCATCAGG-3′) and Pd (5′-CACAGGGAGAGGATGGAGACC-3′) for the p.R237W mutation (PvuII digestion). Digestion products were analyzed by electrophoresis on a 6% acrylamide gel stained with ethidium bromide.

### Plasmid constructs

To generate the different plasmids encoding the GHSR mutations necessary for subsequent functional assays, the previously described GHSR isoform 1a wild-type expression plasmids [GHSR-WT and HA-GHSR-WT (16), designated pGHSR_WT and pHA-GHSR_WT in the current study] were used as templates for site-directed mutagenesis (QuickChange kit; Stratagene, La Jolla, CA). These experiments were performed by means of appropriate primer pairs according to the manufacturer recommendations to introduce the G-to-A or the A-to-T substitution found in the patient, predicting the p.W2X nonsense mutation or the p.R237W missense mutation, respectively. The resulting plasmids were designated pGHSR_W2X, pHA-GHSR_W2X, pGHSR_R237W, and pHA-GHSR_R237W.

### GHSR transcriptional activity and cell surface expression assay

To study the transcriptional activity associated with the different GHSR mutants, HEK293 cells (grown in the absence or on the presence of a ligand) transiently expressing each of the GHSR1a constructs, the isoform encoding the ghrelin receptor (2), or a mock plasmid (pCDNA3.1) were transfected with pSRE-Luc, a commercially available reporter vector (Stratagene)
detecting the serum response element (SRE) pathway. These co-transfection experiments were performed in triplicates in 12-well plates according to the Lipofectamine method (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were incubated for 6 h at 37°C in DMEM medium with 2.5% fetal calf serum with the following conditions: 1) in the absence of ligand, 2) in the presence of the inverse agonist [D-Arg,1 D-Phe,5 D-Trp,7,9 Leu11]-substance P (SPA) (Neosystem, Strasbourg, France) to assess the GHSR constitutive activity, or 3) in the presence of the agonist ghrelin (Neosystem). Luminescence was measured using the Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany) by means of a luciferase assay system (Promega, Madison, WI), and the data of each well were normalized to protein concentration in relative light units per microgram of proteins.

Along with these experiments, when stated, quantification of GHSR expression at the cell surface was performed on additional wells by means of a specific ELISA on cells expressing each of the hemagglutinin (HA)-tagged GHSR constructs, as previously described (16). Briefly, after a fixation step of transfected cells in 4% paraformaldehyde, HA-GHSR molecules expressed at the cell surface were detected by a colorimetric reaction based on horseradish peroxidase-conjugated anti-HA goat polyclonal antibody (Bethyl Laboratories, Montgomery, TX).

**Generation of HEK293 clones stably expressing GHSR_R237W**

To generate clones stably expressing the GHSR carrying the p.R237W mutation, HEK293 cells were transfected with pGHSR_R237W by the Lipofectamine method (Invitrogen). The R237W5 clone was selected on the basis of its binding properties to [125I-His9]ghrelin (1-28) that are similar to those of the wild-type GHSR WT9, a previously described clone expressing the wild-type GHSR (16).

**125I-ghrelin displacement studies**

Membranes of transiently GHSR expressing COS-7 were incubated with 50 pM [125I-His9]ghrelin (1-28) at 2000 Ci/mmol (Amersham, Les Ulis, France) over 1 h at room temperature within 1% BSA binding buffer. Binding from unbound radiolabeled ligand was separated by filtration on 0.22-μm filters (Millipore, Bedford, MA). The total binding was displaced by means of increasing doses of cold ghrelin up to 50 nM (specific binding >90% of total binding).

**Calcium flux assay**

To evaluate the ligand-mediated GHSR activation via the phospholipase C pathway, the intracellular Ca2+ release was measured by means of a Ca2+-sensitive fluorescent reporter (Fluo 4AM; Molecular Probes, Eugene, OR) on R237W5 or WT9 HEK293 clones, as previously reported (16). Briefly, stable clones were incubated with 4 μM of Fluo 4AM for 1 h at loading buffer. Once washed, loaded cells distributed within 96-well black microplates were stimulated with a range of ghrelin concentrations.

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**TABLE 1. Phenotypic features of the propositus (bold characters) and the family members**

<table>
<thead>
<tr>
<th>Family members</th>
<th>I1</th>
<th>I2</th>
<th>II1</th>
<th>II2</th>
<th>II3</th>
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<td>M</td>
<td>M</td>
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<td>35</td>
<td>6/12</td>
<td>10 6/12</td>
<td>4/12</td>
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<td>BMI (kg/m²)</td>
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<td>25.6</td>
<td>13.9</td>
<td>14.5</td>
<td>13.9</td>
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<tr>
<td>SD</td>
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<td>-0.3</td>
<td>-2.0</td>
<td>-1.4</td>
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<td>GH peak (ng/ml)</td>
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<td>IGF-I (ng/ml)</td>
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<tr>
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<td>562</td>
<td>738</td>
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<td>Acylated ghrelin (pg/ml)</td>
<td>287</td>
<td>191</td>
<td>144</td>
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<td>a Before GH treatment.</td>
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<td>b Normal range for pubertal individuals: 336–3320 pg/ml (16).</td>
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<td>c Ghrelin assay performed during GH treatment.</td>
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<tr>
<td>d Normal range for prepubertal individuals: 22–889 pg/ml (16).</td>
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<td>e N stands for normal GHSR allele.</td>
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</table>
Data analysis

The estimate of the IC50, EC50, and maximum response values was performed using the Prism 3 software (GraphPad Inc., San Diego, CA).

Results

Disease phenotype

The episodes of abdominal pain, vomiting, hypoglycemia, and ketosis that occurred two to four attacks per year required iv glucose perfusions. In the course of the clinical evaluation (Fig. 2), the patient showed delayed bone age of 1.5 yr at 4 yr 8 months. At the age of 6 yr 8 months, endocrine investigations revealed markedly low levels of IGF-I (Table 1). Furthermore, he displayed a low GH response to two provocative tests (arginin-insulin and ornithine) with GH peaks less than 10 ng/ml (Table 1), with no other pituitary hormone deficiency and therefore consistent with the diagnosis of partial IGHD. The pituitary size, as assessed by magnetic resonance imaging, was found to be subnormal. At the beginning of the GH treatment (at the age of 6 yr 8 months), an IGF-I generation test was performed. IGF-I and IGF binding protein-3 increased from, respectively, 111 ng/ml and 0.99 μg/ml to 156 ng/ml and 1.50 μg/ml at d 6 (GH 0.1 IU/kg · d day 1 to day 5) and to 200 ng/ml and 1.90 μg/ml at day 11 (GH 0.2 IU/kg · d, d 6–10). The GH treatment was pursued at the dose of 0.035 mg/kg · d (seven injections per week). His height, which was 105 cm (2.7 SD below the mean for age) before treatment, reached 124 cm (1.0 SD below the mean for age) in the second year of treatment. The growth velocity was 9.4 cm/yr the first year and 8 cm/yr the second year. The patient showed a delayed puberty starting at the age of 15 yr; a delayed puberty was also reported in his father but not in the second sibling (II2). Currently at the age of 17 yr 4 months, the patient, whose height is 167 cm (0.8 SD below the mean), is still under GH treatment (0.05 mg/kg · d, seven injections per week); his puberty is not yet completed (P4 A4 G3, testis 7 ml) and his bone age is 15 yr (Fig. 2). Finally, during the GH treatment, the recurrent episodes of abdominal pain, vomiting, and ketosis, first noted in childhood, persisted (about four attacks per year) but, however, without hypoglycemia.

Identification of two GHSR mutations

To test for a possible genetic origin of the partial IGHD documented in the proband, we analyzed coding exons and flanking intronic sequences of three candidate genes: GH1, GHRHR, and GHSR. No disease-causing defects were detected in the GH1 and GHRHR genes. However, two new molecular defects were identified in exon 1 of the GHSR gene, both carried in the heterozygous state (Fig. 3A): a G-to-A transition (c.6G>A) that predicts an early termination codon (p.W2X) and a missense mutation (p.R237W). Amino acid conservation at codon 237 throughout evolution. C, Location of the p.R237W mutation on the GHSR molecule.

Recessive inheritance of the disease phenotype

To further confirm the presence of these two GHSR mutations, while studying their intrafamilial segregation, we designed two sets of primers so that each GHSR mutation creates a restriction site on the corresponding PCR product (see Subjects and Methods): XmnI for the p.W2X mutation and PvuII for the p.R237W mutation. As shown...
in Fig. 1, the p.W2X mutation, found in the patient in the heterozygous state, was inherited from the unaffected father (I1). As for the p.R237W mutation, it is inherited from the unaffected mother (I2), who carries this defect in the heterozygous state and who also transmitted it to her second son of normal stature (II2). Taken together, these data show that the propositus is a compound heterozygote for these GHSR defects. The transmission of the IGHD phenotype within the nuclear family is therefore consistent with a recessive mode of inheritance.

Functional analyses

To study the functional consequences of these nonsense and missense GHSR mutations, several in vitro studies were performed. Early nonsense mutations are known to trigger mRNA decay through a surveillance mechanism called nonsense-mediated decay (17). Although the p.W2X mutation could therefore trigger this pathway, the corresponding GHSR mutant was also studied, keeping in mind that a protein generated from an alternate in-frame initiation codon could partially rescue the GHSR function (the next available in-frame initiation codon is located in the region encoding the end of the first transmembrane domain). The functional consequences of each GHSR mutation were first analyzed by means of a reporter assay reflecting the SRE pathway that is known to be activated by the ghrelin receptor inverse agonist SPA (used to unveil constitutive activity). This experiment was performed by cotransferring each well of HEK293 cells with 80 ng of each GHSR expression plasmid or mock plasmid and 60 ng of the pSRE reporter plasmid. The transcriptional activity is expressed in relative light units per microgram of proteins (mean ± s.e.). Shown is a representative experiment of three independent experiments (each performed in triplicate). The inset represents both the specific basal signal and the ghrelin response for each GHSR construct. The specific basal signal is calculated as the raw basal signal minus the mock transfected signal, and the specific ghrelin response is calculated as the raw ghrelin signal minus the raw basal signal.

FIG. 4. Functional consequences of the two identified GHSR mutations on the SRE pathway. Shown are the transcriptional activity induced by mock, GHSR_WT, GHSR_R237W, and GHSR_W2X in the basal state (in the absence of ligand) in response to ghrelin or the ghrelin receptor inverse agonist SPA (used to unveil constitutive activity). This experiment was performed by cotransferring each well of HEK293 cells with 80 ng of each GHSR expression plasmid or mock plasmid and 60 ng of the pSRE reporter plasmid. The transcriptional activity is expressed in relative light units per microgram of proteins (mean ± s.e.). Shown is a representative experiment of three independent experiments (each performed in triplicate). The inset represents both the specific basal signal and the ghrelin response for each GHSR construct.

Results from an abnormal cell surface expression of the receptor and/or from a specific intrinsic defect, we monitored cell surface expression of HA-tagged GHSRs while measuring the transcriptional response on the GHSR-dependent SRE pathway (Fig. 5A), as previously reported (16). In these experiments, performed in the absence of ligand, the GHSR_R237W mutant, although clearly expressed at cell surface (x-axis), showed a net decrease of constitutive activity (y-axis) when compared with the wild-type GHSR, therefore demonstrating an intrinsic defect of this mutant receptor in its ability to signal constitutively. To characterize further the response of the GHSR_R237W mutant to ghrelin, two kinds of experiments were performed. First, the binding properties the GHSR_R237W mutant for 125I-ghrelin were evaluated on total membranes of COS-7 cells transiently expressing this mutant receptor (Fig. 5B), showing a comparable affinity for ghrelin. Second, the monitoring of Ca2+ release through the Gq pathway, which was assessed in HEK293 cell lines stably expressing the normal or the mutant receptor (Fig. 5C), showed that increasing concentrations of ghrelin resulted in similar dose-response results from an abnormal cell surface expression of the receptor and/or from a specific intrinsic defect, we monitored cell surface expression of HA-tagged GHSRs while measuring the transcriptional response on the GHSR-dependent SRE pathway (Fig. 5A), as previously reported (16). In these experiments, performed in the absence of ligand, the GHSR_R237W mutant, although clearly expressed at cell surface (x-axis), showed a net decrease of constitutive activity (y-axis) when compared with the wild-type GHSR, therefore demonstrating an intrinsic defect of this mutant receptor in its ability to signal constitutively. To characterize further the response of the GHSR_R237W mutant to ghrelin, two kinds of experiments were performed. First, the binding properties the GHSR_R237W mutant for 125I-ghrelin were evaluated on total membranes of COS-7 cells transiently expressing this mutant receptor and compared, in displacement experiments with cold ghrelin, with those of the wild-type receptor expressed in the same conditions. As shown in Fig. 5B, the GHSR_R237W mutant and wild-type GHSR display a similar binding pattern (IC50 0.5 and 0.6 nM for GHSR_WT and GHSR_R237W, respectively), demonstrating a comparable affinity for ghrelin. Second, the monitoring of Ca2+ release through the Gq pathway, which was assessed in HEK293 cell lines stably expressing the normal or the mutant receptor (Fig. 5C), showed that increasing concentrations of ghrelin resulted in similar dose-response
curves (EC50 1.7 and 2.7 nM for GHSR_WT and GHSR_R237W, respectively). Overall, these data show that the p.R237W mutation results in a very subtle defect of the ghrelin receptor, characterized by a partial loss of constitutive activity, whereas both its ability to respond to ghrelin and its cell surface expression are preserved.

Discussion

We report here a case of partial GH deficiency in a patient carrying two heterozygous genetic defects in the ghrelin receptor: a nucleotide substitution predicting a very early termination codon (p.W2X) and a missense mutation (p.R237W). Each of these defects was inherited from a healthy parent. The propositus is the sole compound heterozygote for GHSR defects; he is also the sole individual of short stature within the nuclear family. His young brother, who carries the p.R237W mutation in the heterozygous state, is of normal stature. These observations are therefore consistent with a recessive transmission of the GH deficiency associated with those two GHSR defects, whereas in a previous report on the phenotype of patients with the p.A204E mutation of the GHSR, the short stature was transmitted in a dominant manner (16). In addition, in the current case (patient with the p.W2X/p.R237W genotype), it is noteworthy that the postnatal growth delay combines both typical and atypical features of partial IGHD. The typical features are delayed bone age, marked growth retardation (close to 3.0 SD below the mean for age), low IGF-I levels at baseline (−2.0 SD), two abnormal GH responses to provocative tests (6.0 and 7.0 ng/ml), and a good response to the GH treatment, with positive IGF-I and IGF binding protein-3 generation tests and a significant increase of linear growth (from 2.7 SD to 1.0 SD below the mean for age) within 2 yr of treatment. Such features are consistent with the diagnosis of true GH deficiency. The phenotypic features of this patient that are rather atypical in IGHD are the so-far-unexplained association of recurrent episodes of abdominal pain, vomiting, and ketosis associated, in childhood only, with hypoglycemia and the absence of truncal obesity. At first glance, this lean phenotype is all the more surprising, considering that the patient had a daily high-calorie intake (3500 kcal/d).

Overall, these data raise the question of the mechanisms by which the two GHSR defects identified in this patient result in IGHD with no overweight. Although it is always difficult to draw definite conclusions from the study of a single case, it is tempting to propose the following possible explanations. As for the p.W2X mutation, the nature of the mutation (nonsense), its location (very early), and the functional studies are in favor of a loss-of-
In the recent study, the CA of the GHSR was shown to play a key role in somatic growth, and the functional consequences of the p.R237W missense mutation are completely different, and, in some way, much more subtle. As shown here, the GHSR_R237W mutant appears to be normal in terms of cell surface expression, binding affinity to ghrelin, and ability to respond to ghrelin, as assessed by the study of two different downstream signaling cascades. The only detected anomaly is a partial loss of constitutive activity, but this defect alone does not seem to be deleterious in individuals with the p.R237W/N heterozygous genotype (individuals I2 and I2). For the patient, who is compound heterozygous for the p.W2X and p.R237W mutations, it is therefore tempting to speculate that the nonsense mutation, which results in a loss of all GHSR functions, worsens the phenotype associated with the p.R237W mutation. Consistent with this hypothesis, the previously identified p.A204E mutation (16), which results in a disease phenotype transmitted in a dominant manner, is characterized by a complete loss of constitutive activity associated with a decreased cell surface expression of the receptor. Taken together, these observations further support the hypothesis that the constitutive activity of the GHSR plays a key role in somatic growth and that below a certain threshold, a low constitutive activity is detrimental to growth.

As for the lean phenotype of the patient with the p.W2X/p.R237W genotype, it could fit with the orexigenic and adipogenic properties of ghrelin documented on food intake and energy balance (19, 20). However, this case is in contrast with the counterintuitive observations in which several, not all, of the carriers of the GHSR mutations affecting specifically the constitutive activity of the receptor, including the homozygous A204E proband, were shown to be overweight or obese (16, 21). A possible gain of function toward ghrelin response was suggested (21). Of course, the genotypes differ from the present observation; however, some arguments might help to interpret this apparent discrepancy and speculate on the human syndrome associated with GHSR mutations. First, lessons from melanocortin-4 receptor, another constitutively active GPCR, have demonstrated a concordant obesity phenotype segregating with rare human mutations affecting specifically either the CA or ligand response of the receptor (22), therefore arguing against the gain-of-function hypothesis. Second, the CA of the GHSR was recently shown to regulate its spontaneous internalization (23), therefore suggesting that any mutation affecting selectively the CA would also affect the kinetics of ghrelin response by an impairment of GHSR cell surface distribution. Third, Gbrl°/− or Ghsr°/− mice models have shown that, apart from the very particular situation of animals placed on an early high-fat diet (after weaning), in which the animals have shown a significant degree of protection against obesity (24, 25), possibly due to a developmental issue (25, 26), all different models were not protected against obesity when animals are placed on a high-fat diet at adulthood (12, 13, 27). Altogether one might speculate that the rare GHSR mutations depicted so far are partial or complete loss-of-function mutations and that many possible mechanisms including developmental, genetic, and environmental might compensate the ghrelin system deficiency; a larger sample will critically be required to draw reliable genotype-phenotype correlations.

The proportion of IGHD related to genetic causes is believed to vary between 3 and 30% (28). The identification of rare genetic defects in the genes of the somatotropic axis associated with familial cases of IGHD provided insights on the somatotropic function in humans. Until recently, only two molecular etiologies were documented: 1) mutations located in the GH1 gene itself, recapitulating either partial or complete IGHD transmitted on a recessive or a dominant mode, depending on the defect, and 2) mutations located in the GHRHR transmitted on a recessive mode (GH deficiency type 1B), associating an IGHD and anterior pituitary hypoplasia (29). The present observation therefore together with our previous report (16) provide evidence of a new molecular etiology for IGHDs transmitted on a dominant mode with incomplete penetrance or recessive mode.

Altogether the first case of recessive partial IGHD associated with GHSR mutations, that argues against haploinsufficiency as a disease mechanism, further strengthens the body of evidence (9, 16, 30–32), supporting the physiological role of the ghrelin system in somatic growth.

Acknowledgments

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References