Growth Hormone (GH)-Releasing Hormone Increases the Expression of the Dominant-Negative GH Isoform in Cases of Isolated GH Deficiency due to GH Splice-Site Mutations

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An autosomal dominant form of isolated GH deficiency (IGHD II) can result from heterozygous splice site mutations that weaken recognition of exon 3 leading to aberrant splicing of *GH-1* transcripts and production of a dominant-negative 17.5-kDa GH isoform. Previous studies suggested that the extent of missplicing varies with different mutations and the level of GH expression and/or secretion. To study this, *wt*-hGH and/or different hGH-splice site mutants (GH-IVS+2, GH-IVS+6, GH-ISE+28) were transfected in rat pituitary cells expressing human GHRH receptor (GC-GHRHR). Upon GHRH stimulation, GC-GHRHR cells coexpressing *wt*-hGH and each of the mutants displayed reduced hGH secretion and intracellular GH content when compared with cells expressing only *wt*-hGH, confirming the dominant-negative effect of 17.5-kDa isoform on the secretion of 22-kDa GH. Furthermore, increased amount of 17.5-kDa isoform produced after GHRH stimulation in cells expressing GH-splice site mutants reduced production of endogenous rat GH, which was not observed after GHRH-induced increase in *wt*-hGH. In conclusion, our results support the hypothesis that after GHRH stimulation, the severity of IGHD II depends on the position of splice site mutation leading to the production of increasing amounts of 17.5-kDa protein, which reduces the storage and secretion of *wt*-GH in the most severely affected cases. Due to the absence of GH and IGF-I-negative feedback in IGHD II, a chronic up-regulation of GHRH would lead to an increased stimulatory drive to somatotrophs to produce more 17.5-kDa GH from the severest mutant alleles, thereby accelerating autodestruction of somatotrophs in a vicious cycle. (***Endocrinology* 151: 2650–2658, 2010)**

Human pituitary GH (hGH) contains five exons and four introns and is mainly expressed in the anterior pituitary gland. The 22-kDa GH isoform comprises all five exons that are spliced together to encode the major biologically active form (1, 2). Activation of an in-frame cryptic splice site within exon 3 deletes amino acids 32–46 and generates a truncated 20-kDa isoform representing 5–10% of circulating GH (3–5). Complete skipping of exon 3 generates a 17.5-kDa isoform, which lacks amino acids 32–71 (6). Even though the existence of a 17.5-kDa GH isoform at the protein level has never been positively demonstrated in the normal pituitary, the presence of 17.5-kDa *GH-1* transcripts in trace amounts has been confirmed in RNA extracted from human pituitary tumor (1, 7).

Autosomal dominant GH deficiency type II (IGHD II) is mainly caused by mutations in *GH-1* gene, which occur in and around exon 3 ultimately leading to aberrant splic-

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Abbreviations: C_t, Threshold cycle; GC-GHRHR, GC cells stably transfected with hGHRHR; ER, endoplasmic reticulum; GHD, GH deficiency; hGH, human GH; hGHRHR, human GHRH receptor; hIL-6, human IL-6; IGHD II, autosomal dominant form of isolated GH deficiency; IVS, intervening sequence; rGH, rat GH.

ing of *GH-1* transcripts. Most of the known mutations reported to cause IGHD II reside at the donor splice site within the first 6 bp in intervening sequence 3 (5′-IVS-3) (8) and within the purine-rich sequences in the exon splice enhancer or in the intron splice enhancers (9–14). These splice site mutations reduce the amount of 22-kDa GH transcripts and an increase in the amount of the 17.5-kDa GH isoform (6).

Interestingly, significant variation in the severity and age of onset is observed in GH deficiency (GHD) patients (15, 16), but the reason for this variability remains unclear. IGHD II patients harboring splice site mutations demonstrated on average an earlier age of onset and greater clinical severity than the patients with missense mutations (15). Also, the height reduction in patients with exon splice enhancer mutations is generally less severe than intron-3 splice site mutations but still more severe than missense mutations (12, 15).

Without sequences corresponding to exon 3, the 17.5-kDa GH protein lacks the linker domain between the first two helices of GH molecule including the internal disulfide bridge, thereby disrupting the structure of GH protein (17). This 17.5-kDa GH exerts a dominant-negative effect on the secretion of the 22-kDa GH isoform and reduces its stability (18–20). Furthermore, it has been shown that the 17.5-kDa isoform is retained in the endoplasmic reticulum, disrupts the Golgi apparatus, and impairs secretory protein trafficking (21).

The situation is more complex *in vivo* because GH normally regulates its own expression and production by both direct and indirect (via IGF-I) feedback on two opposing hypothalamic regulatory peptides, GHRH and somatostatin. The GHD in IGHD II reduces negative feedback, resulting in a chronic up-regulation of GHRH and reduction of somatostatin expression (20). This leads to an increased stimulatory drive to GH expression, both from the normal and mutant GH alleles. In mice expressing a high-copy number GH-IVS+1 mutant transgene (producing exclusively 17.5-kDa hGH isoform) mouse GH was greatly reduced, and there was disruption of the somatotroph secretory pathway and the mice were dwarfed (20). Moreover, large numbers of infiltrating macrophages were observed in the pituitaries of these mice, which showed almost total loss of somatotrophs and marked anterior pituitary gland hypoplasia associated with deficits in other pituitary-derived hormones (13, 20). This secondary phenotype was copy number dependent: prolactin and TSH (and LH in males) were significantly reduced in adult high-copy animals, whereas these deficits were milder in low-copy transgenics. Furthermore, the onset and severity of IGHD II were also transgene copy number dependent; high-copy lines rapidly developed profound GHD by weaning, whereas low-copy mice showed milder adult-onset GHD.

A multicenter clinical study performed on 57 subjects from 19 families clearly confirmed the presence of clinical variability in the severity of the IGHD II phenotype, which directly depends on the *GH-1* gene alterations (22). These clinical data were supported by an *in vitro* splicing study showing that with incipient GHD, the gradual increase in expression resulting from loss of GH feedback increases only the proportions of exon 3-skipped transcripts (17.5 kDa isoform) from a mutated allele but not from *wt* allele, the extent of which is dependent on the severity of the mutation in the resting state. The importance of this is that even mutations that have only a mild effect under resting conditions can have their impact amplified *in vivo* because GHD will induce increased expression of 17.5 kDa, in a vicious cycle (23).

The main physiological drive to this increased GH expression is GHRH, so we aimed to model this mechanism *in vitro*. Accordingly, in the present study, we used a rat pituitary cell line (GC cells) stably expressing human GHRH receptor (hGHRHR), in which we could additionally transfect either *wt*-hGH or three different exon 3 splicing mutations (GH-IVS+2, GH-IVS+6, and GH-ISE+28) known to cause variable IGHD II in patients. We could then test whether the stimulation with GHRH (10 nM) alters the relative amounts of different GH isoforms produced and secreted. By using rat GC cells, we could also investigate the impact of the various spice site mutations on the endogenous rat GH (rGH) production. We also used a proteasome inhibitor to assess the impact of different mutations on the amounts of the 17.5-kDa GH isoform accumulating for degradation by the proteasome pathway.

Materials and Methods

Cell culture and treatment

Rat GC cells stably transfected with hGHRHR (GC-GHRHR) cells (a gift from Dr. Paul Le Tissier, National Institute for Medical Research, London, UK) and rat GC-hGHRHR L144H (GC-GHRHR L144H cells) were cultured in DMEM (4.5 g/liter glucose) supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, 10 mM Na-Pyruvate (Life Technologies, Invitrogen AG, Basel, Switzerland), and 100 U/liter penicillin/streptomycin.

Expression vectors and transfection

Human wild-type GH (*wt*-hGH, full-length accession no. J03071) was cloned in pXGH5 (Nichols Institute Diagnostics, San Clemente, CA) as previously described (12), and GH mutants (5′IVS3 + 2 bp, 5′IVS3 + 6 bp, and ISE+28) were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene AG, Basel, Switzerland)

(23). For the studies in GH-expressing cells, the mouse metallothionein-1 promoter regulating the expression of hGH in pXGH5 constructs was replaced with a 555-bp long fragment of the human GH promoter located directly upstream from the transcriptional start site. This GH promoter fragment was amplified from pGL3-hp#1 (24) by PCR and subcloned in pXGH5 (*5′-Hin*dIII, *3′-Bam*HI) to generate the *wt*-hGH, GH-IVS+2, GH-IVS+6, and GH-ISE+28 constructs used here.

Human IL-6 (hIL-6) was amplified from pBMGNeo-IL-6 (gift from Professor S. Rose-John, Department of Biochemistry, Christian-Albrechts-Universität, Kiel, Germany) and cloned downstream of a 600-bp hIL-6 promoter fragment in pGL3 vector. Using *Eco*RI restriction sites, this hIL-6 construct was subcloned in pXGH5 in place of the hGH construct and used as a control in this study.

GC-GHRHR or GC-GHRHR L144H cells were transiently transfected with *wt*-hGH, GH-IVS+2, GH-IVS+6, or GH-ISE+28 using nucleofection (Nucleofector; Amaxa Biosystems GmbH, Cologne, Germany) according to the manufacturer's protocol. GC-GHRHR cells were also transfected with empty vector (puc18) as a negative control. Transfection efficiency was checked using an EGFP-N1control plasmid expressing enhanced green fluorescent protein and analyzed by fluorescence microscopy.

GHRH stimulation and MG 132 treatment

Six hours after transfection, cells were washed in PBS, transferred in OPTIMEM medium (Life Technologies, Invitrogen AG) (without fetal calf serum), and either stimulated or not stimulated with 10 nM GHRH [(Nle^{27})GHRH-(1-29); Bachem AG, Bubendorf, Switzerland] and treated or not with 10 μM MG 132 proteasome inhibitor (Sigma Aldrich, Poole, UK) for 24 h. GC-GHRHR cells transfected with empty vector and stimulated with 10 nM GHRH were used as a negative control.

RNA isolation and real-time PCR (*TaqMan*)

Total RNA was extracted from GC-GHRHR cells 16 h after transfection, using QIAGEN RNeasy kit (QIAGEN AG, Basel, Switzerland) including deoxyribonuclease treatment. Total RNA was reverse transcribed (1 μg total RNA) in 25 μl reverse transcriptase reaction using oligo(deoxythymidine)18 primers, 20 mM of each deoxynucleotide triphosphate, 10× first-strand buffer solution, Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany), and diethyl pyrocarbonate. Primers and probes were chosen and purchased from the predeveloped primer and probe lists of PE Applied Biosystems (Rotkreuz ZG, Switzerland) (22 kDa: Hs00236859-m1; 17.5 kDa, Hs00737954-g1 and 18S: Hs99999901-s1). The PCR universal master mix was also purchased from PE Applied Biosystems. The PCR mixture (25 μl total volume) consisted of primers (forward and reverse 900 nM), TaqMan probe (250 nM), and TaqMan universal master mix (12.5 μl of 2× reaction mixture). The gene sequences were amplified within the cycler (TaqMan; PerkinElmer, Rotkreuz, Switzerland) using the gene-specific oligonucleotide primers. Negative controls included no template control and RNA control to check for genomic contamination. The 18S rRNA was used to normalize cDNA concentrations. Furthermore, relative amounts of splice variants mRNA expression were calculated with the threshold cycle (C_t) and the comparative C_t method was used for relative quantification as described (25) after confirming that *wt*-GH, GH-splice site mutants, and 18S were amplified with the same efficiency. All experiments were performed at least in triplicate.

Western blot analysis

Cellular proteins were extracted 24 h after GHRH stimulation from GC-GHRHR cells using RIPA lysis buffer; 50 μg of total cell lysates were separated on 15% SDS-PAGE gel and blotted on Immobilon P transfer membranes (Millipore, Bedford, MA) using a Trans-Blot semidry apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with polyclonal rabbit antihuman GH antibodies (ICN Pharmaceuticals, Inc., Eschwege, Germany), monoclonal mouse antirat GH (R&D Systems, Abingdon, UK), or polyclonal rabbit antihuman IL-6 (Santa Cruz Biotechnology, Labforce AG, Nunningen, Switzerland). For secondary antibodies, antirabbit immunoglobulin (DakoCytomation, Glostrup, Denmark) or antimouse (Santa Cruz Biotechnology, Labforce AG) was used. Protein bands were visualized by enhanced chemiluminescence substrate reagent and exposed on ECL Plus films (Amersham Pharmacia Biotech, Dübendorf, Switzerland).

GH secretion/production after GHRH stimulation

GC-GHRHR cells were transfected individually with either *wt*-hGH (*wt*-GH or *wt*/puc18) or different mutants (GH-IVS+2, GH-IVS+6, GH-ISE+28) or cotransfected with *wt*-hGH and each of the mutants (*wt*/IVS+2, *wt*/IVS+6, and *wt*/ISE+28). puc18 (empty vector) was used as a negative control. The extracellular GH concentration was measured in aliquots of culture medium and intracellular GH contents were measured in lysed cell extracts from GHRH-stimulated and nonstimulated cells (w/o MG 132 treatment) collected 24 h after GHRH stimulation, using the DSL-10–1900 *Active* hGH ELISA kit (Diagnostics Systems Laboratories, Webster, TX). The results of GH secretion and intracellular GH production were normalized to a total protein content. hGH immunochemiluminometric assays and Diagnostics Systems Laboratories GH ELISAs were performed as previously described (26).

Statistical analysis

Statistical analyses were performed using ANOVA one-way test plus Dunnett's multiple comparison tests comparing each mutant to *wt*-GH. $P < 0.05$ was considered as significant.

Results

Production of *wt*-GH and different GH-splice site mutants expressed in GC-GHRHR cells after GHRH stimulation

After transfection with *wt*-hGH and GH-splice site mutants (GH-IVS+2, GH-IVS+6, GH-ISE+28) in GC-GHRHR cells, the mRNA transcripts coding for 22- and 17.5-kDa hGH were analyzed by quantitative real-time PCR (TaqMan) to determine the relative increase of the specific RNA's under nonstimulated *vs.* GHRH-stimulated conditions (Table 1). Our results showed a significant indi-

TABLE 1. Relative values of GHRH-stimulated *wt*-GH and/or GH-splice site mutant mRNA expression compared with unstimulated values (individual percent fold increase of 22- and 17.5-kDa GH isoforms)

GHRH	22-kDa GH	17.5-kDa GH
10 nM GHRH		
wt-GH	89.2 ± 4.9[a]	3.3 ± 1.8
GH-IVS+2	18.6 ± 1.9[b]	67.1 ± 4.6[a]
GH-IVS+6	24.8 ± 2.2[b]	41.7 ± 3.9[a]
GH-ISE+28	29.5 ± 2.4[b]	38.5 ± 3.3[a]
10 nM GHRH		
wt/*wt*	92.2 ± 4.3[a]	3.7 ± 1.2
wt/IVS+2	57.2 ± 4.7[a]	43.2 ± 3.9[a]
wt/IVS+6	61.4 ± 4.9[a]	37.2 ± 4.1[a]
wt/ISE+28	74.8 ± 5.1[a]	29.1 ± 3.2[a]

The 18S rRNA was used to normalize cDNA concentration. The levels of expression were calculated by comparative C_t method relative to 18S. Results are given as individual percent fold (percentage) increase of 22- and 17.5-kDa GH isoforms in GC-GHRHR cells expressing *wt*-GH and/or GH-splice site mutants and were compared with unstimulated conditions. Data represent the means ± SD of at least three independent experiments performed in duplicates.

[a] $P < 0.001$.

[b] $P < 0.01$.

vidual percent fold increase of both the 22- and 17.5-kDa GH isoforms, which directly depended on the combination of the various GH variants expressed (*wt*-GH, GH-IVS+2, GH-IVS+6, GH-ISE+28). Moreover, we observed an overall increase in mRNA production for *wt*-hGH, GH-IVS+2, GH-IVS+6, and GH-ISE+28 of 56, 42, 41, and 40% when compared with nonstimulated conditions as determined by RT-PCR (Supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Next, we investigated whether the increase we observed in mRNA levels after GHRH stimulation is followed by a similar increase at the protein level. The 17.5-kDa GH isoform is targeted for degradation by proteasome pathway, so to assess the production of this isoform, some cells were also treated with a proteasome inhibitor (MG 132). When expressed alone, the *wt*-hGH and GH-splice site constructs produced an increase in protein expression of both 22- and 17.5-kDa GH isoforms after GHRH stimulation (Fig. 1). Additional treatment with proteasome inhibitor resulted in a higher expression of the 17.5-kDa isoform, especially for GH-IVS+2 and GH-IVS+6.

In similar experiments, we also analyzed the effect of GHRH stimulation when the GH-splice site mutants were coexpressed with *wt*-hGH in GC-GHRHR cells. The data of the real-time PCR experiments highlighting the relative individual percent fold increase of the 22- and 17.5-kDa GH isoform are shown in Table 1. The relative values of GHRH stimulated *wt*-GH and/or GH-splice site mutants mRNA expression were compared with the unstimulated

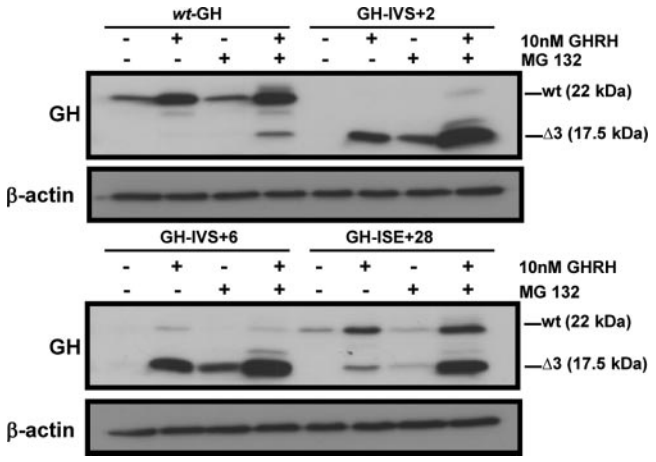

FIG. 1. GHRH-stimulated production of *wt*-hGH and GH-splice site mutants expressed in GC-GHRHR cells. Western blot analysis of the splicing pattern in GHRH-stimulated *vs.* nonstimulated conditions including additional treatment with MG 132 proteasome inhibitor (as described in *Materials and Methods*). These experiments were repeated at least three times.

values and found to mirror the splice site activity of the specific GH mutant isoforms (Table 1).

Furthermore, RT-PCR analysis revealed that *wt*/*wt*, *wt*/IVS+2, *wt*/IVS+6, and *wt*/ISE+28 display a total increase in mRNA productions of 50, 40, 41, and 53% when compared with nonstimulated conditions (Supplemental Fig. 2). As before, we observed an overall increase in GH expression after GHRH stimulation, which was even more pronounced for the 17.5-kDa isoform, in cells expressing *wt*/IVS+2, *wt*/IVS+6, and *wt*/ISE+28 after additional treatment with MG 132 (Fig. 2).

To show that GHRH stimulation was acting via the GHRHR and not by an unspecific pathway, we tested a

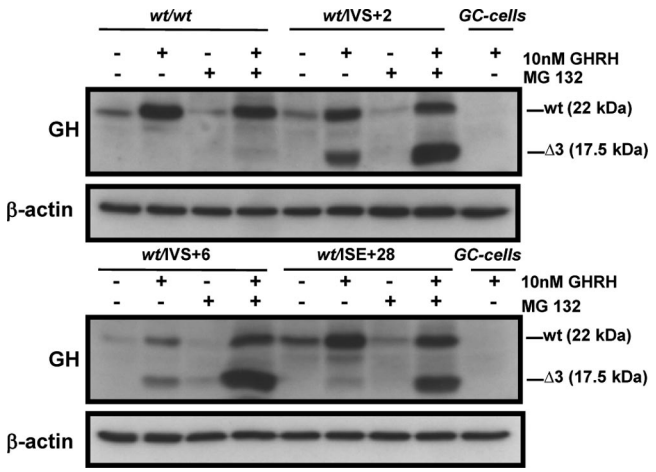

FIG. 2. Expression and analysis of different GH isoforms in GHRH-stimulated GC-GHRHR cells concomitantly expressing *wt*-GH and GH-splice site mutants. The splicing pattern in GHRH-stimulated *vs.* nonstimulated conditions including additional treatment with MG 132 proteasome inhibitor analyzed by Western blot (as described in *Materials and Methods*). This experiment was repeated at least three times and representative blots are shown.

mutant GHRHR (GC-GHRHR L144H), known to exhibit impaired GHRH signaling. When GC-GHRHR L144H cells were transfected with *wt*-hGH, GH-IVS+2, GH-IVS+6, and GH-ISE+28 and stimulated with 10 nM GHRH, no increase in protein expression could be detected after GHRH stimulation for any of the GH variants tested (Fig. 3A). We also tested whether the expression of hIL-6, another cAMP-responsive target gene, would be increased after GHRH stimulation. However, 10 nM GHRH treatment did not increase hIL-6 expression from transfected GC-GHRHR cells (Fig. 3B).

The secretion of GH from GC-GHRHR cells after GHRH stimulation

Next, we compared the secretion into the culture medium of these mutants with that of the *wt*-hGH after GHRH stimulation (Fig. 4). All three GH-splice site mutants (GH-IVS+2, GH-IVS+6, or GH-ISE+28) secreted significantly lower amounts of GH ($P < 0.01$) compared with *wt*-hGH (Fig. 4A). Moreover, GHRH stimulation of GC-GHRHR cells coexpressing of *wt*-hGH and GH-IVS+2 or GH-IVS+6 (*wt*/IVS+2, *wt*/IVS+6) resulted in significantly reduced GH secretion ($P < 0.01$, Fig. 4C) compared with cells expressing *wt*-hGH alone (*wt*/puc18), confirming the dominant-negative impact of GH-IVS+2 and GH-IVS+6 on the secretion of *wt*-hGH in this system.

Intracellular GH content after GHRH stimulation

Intracellular GH production was measured under the same experimental conditions. GC-GHRHR cells expressing GH-IVS+2, GH-IVS+6, and GH-ISE+28 and stimulated with GHRH displayed significantly reduced intracellular GH contents ($P < 0.01$) when compared with cells expressing only *wt*-hGH (Fig. 4B). Similarly, coexpression of *wt*-hGH and GH-IVS+2, GH-IVS+6, or GH-ISE+28 (Fig. 4D) resulted in significantly reduced intracellular GH contents ($P < 0.01$ for GH-IVS+2 and GH-IVS+6; $P < 0.05$ for GH-ISE+28) when compared with *wt*-hGH (*wt*/puc18). Taken together, these results suggest that that GH-splice site mutants initially generate similar amounts of product in response to GHRH stimulation but that the mutant proteins exert dominant-negative effects on the intracellular storage as well as secretion of both mutant and *wt*-hGH.

Production of endogenous rGH in GC-GHRHR cells expressing different GH-splice site mutants after GHRH stimulation

Most *in vitro* studies have used transfected proteins to investigate dominant-negative effects. An advantage of using rat GC cells is that it was possible to investigate the impact of increased production of 17.5-kDa GH isoform on an endogenously produced rGH. Accordingly, rGH expression was also analyzed by Western blot after *wt*-hGH and hGH-splice site mutants were expressed in GC-GHRHR cells and stimulated with 10 nM GHRH. GC-GHRHR cells transfected with empty vector and stimulated with 10 nM GHRH were used as a control. GC-GHRHR cells expressing *wt*-hGH showed an increase in rGH expression of 13% after GHRH stimulation (Fig. 5) whereas in cells expressing GH-IVS+2, GH-IVS+6, and

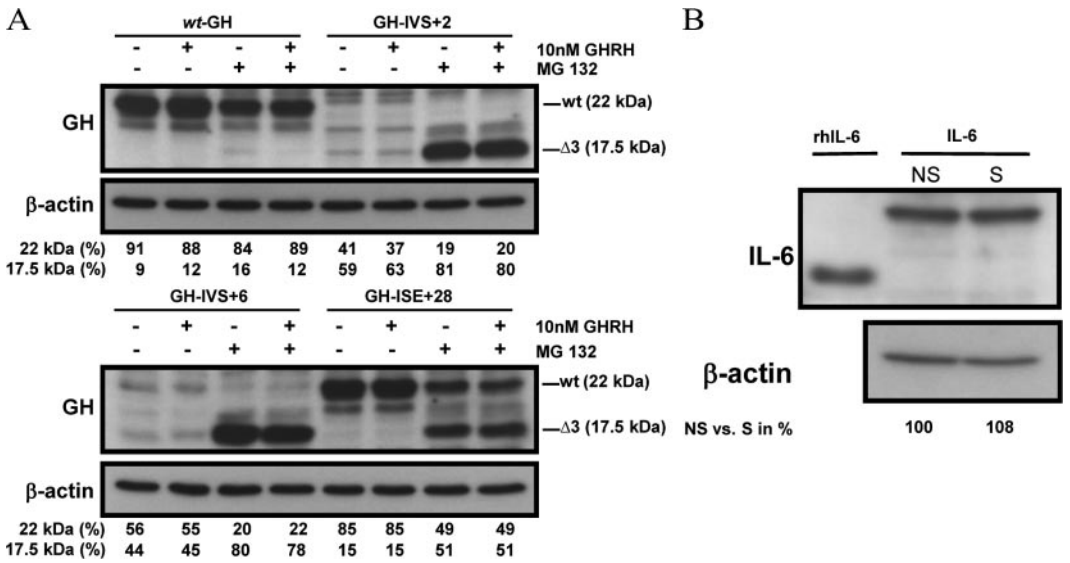

FIG. 3. Transfection of *wt*-hGH and GH-splice site mutants in GC cells expressing nonfunctional GHRHR (L144H) and expression of hIL-6 in GC-GHRHR cells after GHRH stimulation. GC-GHRHR cells harboring L144H mutation were individually transfected with *wt*-hGH, GH-IVS+2, GH-IVS+6, or GH-ISE+28 (A) and GC-GHRHR cells were transfected with hIL-6 (B). In both cases stimulation with 10 nM GHRH was applied for 24 h after Western blot analysis and GHRH-stimulated induction given as percentage of GHRH-stimulated (S) over nonstimulated (NS) and calculated by setting NS conditions as 100%. These experiments were repeated at least three times.

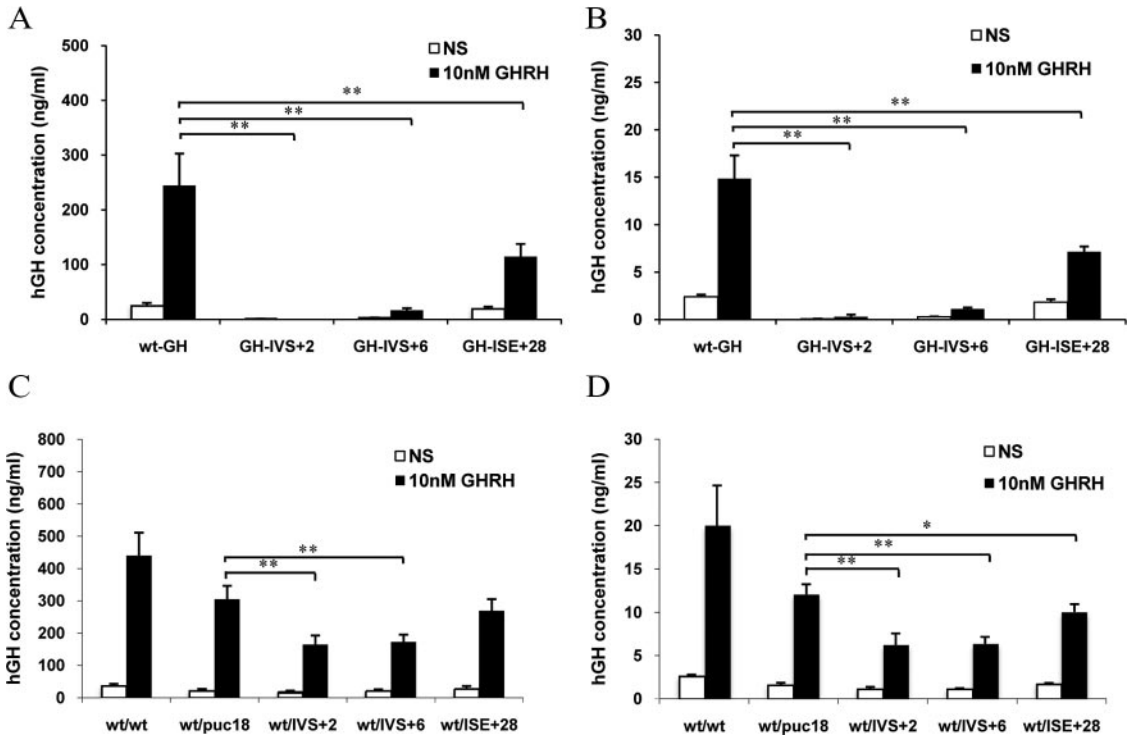

FIG. 4. GH secretion/production after GHRH stimulation. GH secretion was measured in aliquots of culturing medium from GC-GHRHR cells singly transfected with *wt*-hGH, GH-IVS+2, GH-IVS+6, or GH-ISE+28 after 24 h stimulation with GHRH (A). After that, cells were lysed and intracellular GH content was measured in the whole-cell lysates of stimulated as well as nonstimulated (NS) cells (B). Similarly, GH secretion (C) and intracellular GH content (D) were measured for GHRH-stimulated and nonstimulated conditions in cells cotransfected with *wt*-hGH and each of the mutants together. All the values are normalized to total protein content. The results represent a mean ± SD of at four independent quantification experiments. *, $P < 0.05$; **, $P < 0.01$.

GH-ISE+28, rGH levels were reduced by 53, 23, and 13%, respectively. These results show that hGH-splice site mutants producing different amounts of 17.5-kDa hGH isoform after GHRH stimulation exert a dominant-negative effect also on endogenous rGH production in GC-GHRHR cells.

Discussion

IGHD II is often associated with dominant-negative mutations located on intron 3 of the *GH-1* gene, by either direct interference at the 5′ splice site or compromising splice enhancer sequences. As a consequence, these mutations cause variable amounts of exon 3 skipping, thereby generating transcripts coding for the 17.5-kDa GH isoform. The presence of 17.5-kDa hGH transcripts in trace amounts has been reported so far only in human pituitary tumor (1, 7), whereas for the normal pituitary, the expression of this isoform on either transcript or protein level has never been demonstrated.

In a previous study, we compared the impact of stimulating GH expression directly by dexamethasone on the production of different GH isoforms from *wt*-hGH and GH-splice site mutants (GH-IVS+2, GH-IVS+6, and GH-ISE+28), relying on the glucocorticoid regulatory element present within intron-1 of *GH-1* gene (23). The results clearly demonstrated that with dexamethasone exposure, stimulation of gene transcription from GH-splice site mutants (but not from *wt*-GH) increased production of the 17.5-kDa GH isoform relative to the 22-kDa isoform exerting a final effect on cell proliferation and apoptosis, which was the most pronounced for

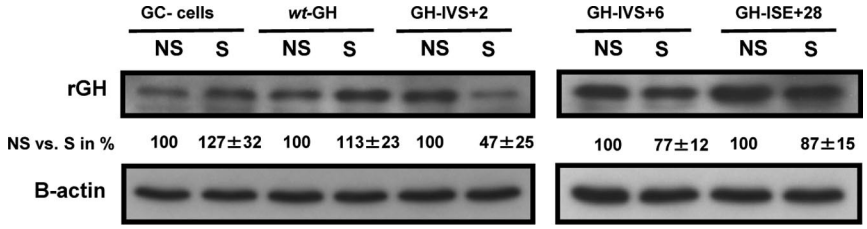

FIG. 5. Effect of *wt*-hGH and GH-splice site mutants on endogenous rGH production. GC-GHRHR cells expressing empty vector, *wt*-hGH, GH-IVS+2, GH-IVS+6, or GH-ISE+28 were stimulated with 10 nM GHRH and the levels of endogenously produced rGH were detected by Western blot. Rat GH was quantified using Quantity One software (Bio-Rad Laboratories) and normalized to β-actin control. The bands corresponding to nonstimulated conditions (NS) were quantified and set to 100% and GHRH-stimulated conditions (S) were compared against this. Data are given as mean ± SD of three independent experiments.

GH-IVS+2. This was consistent with our hypothesis that driving expression would force more misplicing and perhaps overload the proteosomal degradation pathway for the misfolded 17.5-kDa hGH, in proportion to the severity of the mutation. However, the method we used for driving expression was unphysiological and was studied in corticotroph cells to avoid the background of endogenous GH secretion. Furthermore, we did not attempt to distinguish between mechanisms at the RNA, protein storage, or secretory pathway level.

In the present study, we addressed these concerns in several ways. First, we used rat pituitary GC cells that produce endogenous rGH. Rat GC cells express little or no endogenous rat GHRHR and are thus refractory to GHRH stimulation (Robinson, I. C., unpublished data) but when transfected with human GHRHR, show cAMP responses to GHRH (Robinson, I. C., and P. Le Tissier, unpublished data). We could thus transfect different GH-splice site mutants (GH-IVS+2, GH-IVS+6, GH-ISE+28) under the control of a native hGH promoter and stimulate production with GHRH (10 nM). At the transcript level, both *wt*-GH and GH mutants showed an overall increase in mRNA after stimulation. On the other hand, GHRH stimulation evoked an increase in the relative amounts of the 17.5-kDa isoform produced by each of GH-splice site mutants singly expressed or coexpressed with *wt*-GH. Note that this increase varied among different GH-splice site mutants being more pronounced for GH-IVS+2 than for GH-IVS+6 and GH-ISE+28. Taken together, our data obtained at mRNA level suggest that there is no defect or dominant effect at the transcriptional level in the somatotroph environment.

Like any other secretory protein, GH must be folded correctly to exit endoplasmic reticulum (ER) (27). Because the 17.5-kDa GH isoform lacks the entire exon 3, which includes the internal disulfide bridge, the internally truncated protein may well be recognized as a misfolded protein, although it appears to be poorly recognized as a trigger for the unfolded protein responses (21). In general, such proteins are rapidly degraded, and the mechanism for such degradation includes transport of the proteins back across the membrane of ER to the cytosol in which they are degraded by the proteasomal pathway (28, 29). Selective degradation of the 17.5-kDa hGH could alter the amounts stored in somatotrophs, and we addressed this by combining GHRH stimulation with treatment with proteasome inhibitor (MG 132). Western blot data revealed higher expression of the 17.5-kDa isoform in cells singly expressing GH-IVS+2 and stimulated with GHRH and treated with MG 132 when compared with cells expressing GH-IVS+6, GH-ISE+28, or *wt*-hGH. This suggests that the amount of the 17.5-kDa isoform targeted for degradation was higher when GH-IVS+2 was expressed than that in the case of GH-IVS+6 or GH-ISE+28, which is consistent with its more severe effect on splicing and more severe phenotype. In fact, without MG 132 treatment, we were unable to detect any 17.5-kDa protein in cells transfected with GH-splice site mutants and stimulated with GHRH, which supports the idea that misfolded 17.5-kDa GH isoform is translated from its transcript but degraded rapidly thereafter. Under normal conditions, or with only a mild extent of exon 3 skipping, it seems that the degradation pathway is capable of preventing significant dominant-negative effects on *wt*-hGH. However, if this pathway is inhibited (or conversely, if the generation of the 17.5-kDa GH protein is increased), the accumulation of this protein begins to exert a dominant-negative effect on *wt*-hGH.

Various hypotheses for the dominant-negative mechanism have been suggested in numerous studies (13, 18–20, 30), but all agree that a major effect is to block the ability of somatotrophs to store and secrete 22 kDa GH. Furthermore, the accumulation of the 17.5-kDa isoform in cytosol eventually proves toxic to the cells. *In vivo* this eventually leads to a major loss of somatotrophs, which is probably accelerated by invasion of activated macrophages (20). We looked for such a toxic effect of accumulated the 17.5-kDa GH isoform in cells expressing *wt*-hGH and GH-splice site mutants after GHRH stimulation prolonged up to 72 h (with or without MG 132 treatment). We could find no difference in cell proliferation or apoptosis assessed through phosphatidylserine redistribution in annexin V binding assays (fluorescence-activated cell sorter analysis) or through levels of noncleaved and cleaved caspase-3 (Western blot) of cells individually expressing *wt*-hGH, GH-IVS+2, GH-IVS+6, or GH-ISE+28 after the stimulation (data not shown). This may be because in our somatotroph *in vitro* system with transient transfection, the production rate of the 17.5-kDa GH isoform does not exceed the proteasome degradation capacity, so the 17.5-kDa isoform does not accumulate in the cytosol. In addition, it is important to note that somatotroph cells of IGHD II patients are in the terminal stage of differentiation and rarely divide, whereas the GC-GHRHR cells are from a tumor cell line, which constantly divide. McGuinness *et al.* (20) reported toxic effects with an GH-IVS+1 mutant stably transfected into the same parent GC cell line but were nevertheless able to generate a stable line.

On the other hand, after GHRH stimulation our secretion studies demonstrated that GH-splice site mutants *per se* were significantly less secreted and their intracellular content was significantly reduced when compared with *wt*-hGH. The experiments in which *wt*-hGH and the mu-

tants were coexpressed showed that GH-IVS+2 and GH-IVS+6 exerted a dominant-negative effect on *wt*-hGH secretion after GHRH stimulation. GH-ISE+28 tended to reduce *wt*-GH secretion but the difference did not reach statistical significance. Furthermore, all three GH-splice site mutants significantly reduced intracellular production of *wt*-hGH after stimulation. Given the previous reports showing disruption of the secretory granule pathway when *wt*-GH and GH mutants are coexpressed, it seems that if larger amounts of 17.5 kDa remain within the ER in the presence of *wt*-hGH, it prevents efficient packaging, storage, and secretion of all the GH isoforms produced, including *wt*-hGH from the normal allele.

These results are in line with clinical variability observed in patients studied with the same mutations. Our patients with the heterozygous GH-IVS+2 mutation were found to be more likely earlier diagnosed, more severe in progression, and presenting with short stature already at a younger age than the patients carrying the GH-IVS+6 mutation (15) or GH-ISE+28, which presented a mild IGHD II phenotype (Mullis, PE unpublished clinical observations). Moreover, as reported in a multicenter study (22), subjects suffering from IGHD II caused by GH-splice site mutations, especially GH-IVS+2, may also present with other hormonal deficiencies, mainly ACTH and TSH.

In conclusion, by using this *in vitro* model of IGHD II, we provide further evidence that the effects of the 17.5-kDa GH isoform are highly dependent on the amounts produced, which goes some way to explaining the variable severity of phenotype among patients harboring mutations with subtle differences in the amounts of misplicing generated from the mutant allele, in the presence of a normal allele. Small amounts of 17.5 kDa hGH protein can be cleared rapidly by the proteosomal pathway and have little effect within the ER. Where these amounts are increased, *e.g.* by more severe mutations (13), GHRH stimulation, or inhibiting the proteasome, the persistence of increased amounts of 17.5 kDa GH present together with *wt*-hGH interact to reduce GH storage, ultimately compromising GH secretion. *In vivo*, the GHD and lack of GH feedback generates a vicious cycle, increasing the expression of both *wt*-hGH and 17.5-kDA GH isoforms, which ultimately damages the somatotrophs. We suggest that in some IGHD II patients, the loss of negative feedback control regulating normal GH expression and constant endogenous GHRH stimulation of somatotrophs can result in increased amounts of the 17.5-kDa GH isoform to be produced, contributing to the variability in onset, severity, and progression of the disease. Progressive GHD in combination with GHRH up-regulation may accelerate the autodestruction of somatotrophs, which may result in macrophage infiltration in pituitary and destruction of other hormone-producing cell types (20). This could potentially lead to development of other hormonal deficiencies and emphasizes the importance of keeping adult untreated patients under regular observation and/or consideration of continued recombinant hGH treatment in adulthood.

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