A homozygous mutation in growth hormone 1 (GH1) was recently identified in an individual with growth failure. This mutation, c.705G>C, causes replacement of cysteine at position 53 of the 191-amino-acid sequence of 22 kDa human GH (hGH) with serine (p.C53S). This hGH molecule (hereafter referred to as GH-C53S) lacks the disulfide bond between p.Cys-53 and p.Cys-165, which is highly conserved among species. It has been reported previously that monomeric GH-C53S has reduced bioactivity compared with WT GH (GH-WT) because of its decreased ability to bind and activate the GH receptor in vitro. In this study, we discovered that substitution of p.Cys-53 in hGH significantly increased formation of hGH dimers in pituitary cells. We expressed His-tagged hGH variants in the cytoplasm of genetically modified Rosetta-gami B DE3 Escherichia coli cells, facilitating high-yield production. We observed that the bioactivity of monomeric GH-C53S is 25.2% of that of GH-WT and that dimeric GH-C53S–His has no significant bioactivity in cell proliferation assays. We also found that the expression of GH-C53S in pituitary cells deviates from that of GH-WT. GH-C53S was exclusively stained in the Golgi apparatus, and no secretory granules formed for this variant, impairing its stimulated release. In summary, the unpaired Cys-165 in GH-C53S forms a disulfide bond linking two hGH molecules in pituitary cells. We conclude that the GH-C53S dimer is inactive and responsible for the growth failure in the affected individual.

In 2005, Besson et al. (1) characterized a case of isolated growth hormone deficiency in a 9-year-old boy with a height of 116 cm (−3.6 standard deviation score). This patient presented a high basal hGH level (13.5 ng/ml) and high hGH levels after insulin-induced hypoglycemia (44.7 ng/ml). The patient’s low insulin-like growth factor 1 level (38 ng/ml, −3.4 standard deviation score) raised suspicion regarding bioinactive hGH, as described first in 1978 by Kowarski et al. (2). Genetic analysis revealed a homozygous mutation, c.705G>C, in the patient’s GH-1 gene. The consanguineous parents were both heterozygous for c.705G>C and of normal height, indicating a recessive mode of inheritance. Treatment with 30–45 μg of rhGH per kilogram of body weight per day resulted in catch-up growth, and finally a height of −1.6 standard deviation score was reached (1).

The WT hGH molecule contains two disulfide bridges, forming a short C-terminal loop and a long loop between p.Cys-53 and p.Cys-165 that links the crossover connection loop of helices 1 and 2 to helix 4 (3). These disulfide bridges are highly conserved among species and are also found in prolactin (4, 5). On the protein level, c705G>C leads to replacement of cysteine in position 53 by serine, which abolishes the disulfide bond with p.Cys-165 in hGH.

This mutant growth hormone, GH-C53S, has been reported to have reduced affinity to the growth hormone receptor (GHR) and to induce STAT5 activation in the 293-GHR cell luciferase reporter gene assay (6), a method to determine GH’s ability to bind and activate the GHR, with 40.0% of GH-WT’s potency. GH-C53S is thus considered bioinactive at physiological serum levels (1). In line with this, p.C53S-modified bovine GH (bGH-C53S) showed no growth-promoting effect in a transgenic GH-deficient mouse model, although the bGH-C53S levels were as high as the bGH levels in giant mice (7).

Taken together, these findings indicate that p.Cys-53 and its corresponding disulfide bond are essential for biological function of hGH. This is in contrast to well-established experiments showing that reduced alkylated hGH is as biologically active as GH–WT, although it is devoid of both disulfide bridges (8). Furthermore, it is unclear how the moderate reduction in potency of GH-C53S observed in the 293-GHR cell assay causes loss of function of hGH, represented by growth failure and very low IGF-1 levels. In this study, we expressed GH-C53S in the AtT20 and GC (9) pituitary cell lines and conducted further experiments to understand how the GH1 c.705G>C mutations cause growth hormone deficiency.

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![Figure 1. hGH expression in transiently transfected AtT20 cells.](image)

In pituitary AtT-20 cells, secretion of GH-C53S was significantly elevated, whereas the intracellular amount of GH-C53S was significantly diminished compared with GH-WT. For the double mutant GH-C53S/C165A, only the intracellular amount was reduced, whereas secretion was identical to GH-WT (Fig. 1). Coexpression of GH-C53S and GH-WT affected overall hGH concentrations only slightly. Coexpression of C53S-hGH with placental growth factor (PlGF) confirmed that the reduced hGH expression was not caused by differences in transfection efficiency (data not shown). Stimulation of release of secretory granules in hGH-transfected AtT-20 cells using the secretagogue 8-Br-cAMP could only elevate GH release in GH-WT–transfected cells, not in GH-C53S or GH-C53S/C165A–transfected cells (Fig. 2). Immunofluorescent staining of hGH and the Golgi apparatus in transfected AtT-20 cells revealed the absence of secretory granules present in GH-WT–transfected cells (Fig. 3A) for GH-C53S–transfected (Fig. 3B) and GH-C53S/C165A–transfected (Fig. 3C) cells. Furthermore, colocalization of GH-C53S and GH-C53S/C165A with β-COP (a protein associated with the Golgi complex) revealed that both mutants were restricted to the Golgi apparatus.

**GH-C53S forms hGH dimers**

In a Western blot analysis of cell lysates under nonreducing conditions (Fig. 4A) of GH-C53S–transfected AtT20 and GC cells, the substantial part of hGH was at 44 kDa, representing dimeric hGH (Fig. 4A, lanes 10 and 18). Under reducing conditions, GH-C53S appeared at about 22 kDa (Fig. 4B, lanes 10 and 18), indicating that GH-C53S formed an hGH dimer through disulfide bonds. Interestingly, GH-C53S in supernatants of transfected AtT20 and GC cells showed stronger bands at 22 kDa than at 44 kDa (Fig. 4A, lanes 12 and 19), indicating reduced secretion of GH-C53S dimers. The supernatants and lysates from HEK-293 and CHO-K1 cells after immunoprecipitation to increase the concentration (Fig. 4, A and B, lanes I–9) contained mainly lower-molecular-mass 22-kDa GH-C53S, indicating that GH-C53S dimer formation is more prominent in pituitary cells; however, present data are insufficient to conclude that dimer formation occurs exclusively in pituitary cells and not in other mammalian cells. Additional replacement of p.Cys-165 with alanine inhibited formation of 44-kDa hGH (Fig. 4A, lanes 14 and 16), indicating that GH-C53S dimers are linked by disulfide bonds between the p.Cys-165 residues. Monomeric GH-C53S and GH-C53S/C165A under nonreducing conditions migrated slower than GH-WT and appeared as double bands. The lower band of the mutant hGH equaled GH-WT under reducing conditions (Fig. 4B). Both mutants migrated slower than GH-WT under nonreducing conditions, suggesting a less compact structure because of the disrupted disulfide bond.

**GH-C53S is partially O-glycosylated**

The GH-C53S and GH-C53S/C165A mutants showed two closely spaced bands at 24 kDa in addition to the 22-kDa band in all mammalian cell types used in this study (Fig. 4). After purification using affinity chromatography, GH-C53S incubated with neuraminidase and O-glycosidase showed only one band at 24 kDa and one enhanced 22-kDa band (Fig. 5). As O-glycosylation does not occur in most *Escherichia coli* strains (10), both additional 24-kDa bands were not present in GH-C53S generated in Rosetta-gami B DE3 cells (Fig. 6).

**Biological activity of GH variants**

First, we used hGH expressed in CHO cells to determine the bioactivity of the generated monomeric hGH variants by stimulating BaF/B03 cells stably transfected with the GHR and Nb2 cells expressing a truncated prolactin receptor with increasing amounts of hGH variants (Fig. 7, A and B). The results closely matched the activities reported previously by Besson et al. (1); the bioactivity of monomeric GH-C53S is moderately reduced compared with GH-WT. To study the biological properties of the GH-C53S dimer, we needed to synthesize the molecule in greater quantities than what was achievable in pituitary cell lines. To overcome this problem, we developed a protocol to express hGH and the GH-C53S mutant correctly folded, including the disulfide bonds in *E. coli*. We employed Rosetta-gami B DE3 *E. coli* cells that carry mutations in thioredoxin reductase (trxB) and GSH reductase (GOR), permitting formation of disulfide bonds in the cytoplasm. This enabled high-yield synthesis of His-tagged GH-WT–His and the GH-C53S–His dimer in the range of milligrams per liter. Interestingly, hGH expression in the periplasm of *E. coli* was not effective in generating sufficient amounts of hGH. Following purification using nickel-nitrilotriacetic acid–agarose chromatography, immunoaffinity chromatography and size-exclusion chromatography, we stimulated BaF/B03 cells stably transfected with the GHR with increasing concentrations of hGH variants and measured cell proliferation to assess bioactivity (Fig. 8). Comparing EC₅₀ values, we found that monomeric GH-C53S–His
had 12.6% of GH-WT–His potency. In contrast, dimeric GH-C53S–His had 0.011% of GH-WT–His potency.

The GH-C53S dimer does not bind to the GHR

To investigate the underlying cause of this dramatic loss of bioactivity, we studied the hGH variants’ affinity to the GHR using a growth hormone–binding protein (GHBP) competition assay (Fig. 9). Using the mutant hGH generated in E. coli, we found that GH-WT–His affinity to the GHR was comparable with the reference standard 80/505, whereas the GH-C53S–His monomer showed 25.2% of GH-WT–His affinity. The GH-C53S–His dimer, however, had no detectable affinity to the GHR.
The GH-C53S dimer features elevated protein stability

As protein stability is another important factor determining the biological half-life of hormones, we studied the resistance of the hGH variants to trypsin degradation. Monomeric GH-C53S–His was as stable as GH-WT–His (Fig. 10, A and B). As shown by the stronger bands at the 30- and 60-min time points (Fig. 10C), dimeric GH-C53S–His was less susceptible to trypsin degradation than GH-WT–His.
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Discussions

Our analysis revealed that replacement of cysteine 53 with another amino acid in hGH induced formation of hGH dimers. The formation of dimers was most prominent in pituitary cells. The absence of dimer formation following replacement of cysteine 53 with another amino acid in hGH induced formation of hGH dimers. The formation of dimers was most prominent in pituitary cells.

Figure 8. Cell proliferation assay with increasing concentrations of hGH-His variants purified from Rosetta-gami B cells stimulating stably GHR-transfected BaF/B03 cells. Results are expressed as luminosity (corresponding to the number of viable cells) and represent the mean and S.D. of three separate experiments performed in duplicate. EC_{50} concentrations and 95% CI: GH-WT–His, 0.17 ± 0.019 ng/ml; GH-C53S–His monomer, 1.35 ± 0.33 ng/ml; GH-C53S–His dimer, ~1500 ng/ml; compared with NIBSC 80/505, 0.045 ± 0.05 ng/ml.

Figure 9. Competitive GHBP binding assay of hGH-His generated in Rosetta-gami B DE3 cells. Shown is displacement of rhGH-b through increasing amounts of hGH variants. Fluorescence measures the amount of non-displaced rhGH-b. Error bars represent the S.D. of two experiments performed in duplicates. IC_{50} concentrations: GH-WT–His, 71.4 ng/ml; pituitary-derived NIBSC reference preparation 80/505, 75.0 ng/ml; GH-C53S–His monomer, 283.5 ng/ml; p < 0.01. IC_{50} values for the C53S-hGH–His dimer could not be calculated because of lack of displacement.

Table

| Time (min) | 0 | 15 | 30 | 60 | 120 |
|-----------|---|----|----|----|-----|
| Trypsin   | - | +  | +  | +  | +   |
| A GH-wt-his | - | -  | -  | -  | -   |
| B GH-C53S–His | - | -  | -  | -  | -   |
| C GH-C53S–His | - | -  | -  | -  | -   |

Figure 10. Tryptic digestion of hGH mutants generated in Rosetta-gami B DE3 cells. A–C, Western blots of GH-WT–His (A), GH-C53S–His monomer (B), and GH-C53S–His dimer (C) after incubation with trypsin or water for 0–120 min.

Discussion

Our analysis revealed that replacement of cysteine 53 with another amino acid in hGH induced formation of hGH dimers. The formation of dimers was most prominent in pituitary cells. The absence of dimer formation following replacement of cysteine 53 with another amino acid in hGH induced formation of hGH dimers. The formation of dimers was most prominent in pituitary cells. Under reducing conditions provide evidence that the GH-C53S dimer is held together by an intermolecular disulfide bond between the two p.Cys-165 residues. Physiologically, a small percentage of pituitary-derived hGH (GH-WT) is present as a disulfide-linked dimer and a noncovalently linked dimer, which is also seen in our Western blots. This GH-WT dimer has been reported to have little growth-promoting effect (11), but it remains undefined which cysteines are linked to form the 45-kDa hGH dimer.

It has been known for nearly 50 years that reduction and alkylation of the cysteines in hGH, leading to disruption of the disulfide bonds, retain the growth hormone’s biological activity (8). Further evidence in support of this notion is derived from a number of more recent studies (12–14).

The affinity of predominant monomeric GH-C53S generated in CHO-K1 cells to GHBP was reduced to only 25.1% of GH-WT (data not shown) and to 25.2% of GH-WT for GH-C53S–His generated in E. coli. Alteration of the cysteine’s side chain is expected to be a minor factor in the reduction of binding affinity, as alterations of amino acids with side chains directly interacting with the GHR have shown little effect on binding affinity (15, 16). It has been demonstrated that even strong alterations (up to 30-fold) in binding affinity had no effect on bioactivity in proliferation assays and transgenic mice (17, 18). Consistent with the finding by Besson et al. (1), we found that predominant monomeric GH-C53S produced in CHO-K1 cells in the BaF/B03 assay had 33.3% bioactivity compared with GH-WT.

To study GH-C53S dimers in detail and overcome low expression levels in pituitary cells, we also chose to express this mutant in E. coli. In our study, the oxidative cytoplasm of genetically engineered Rosetta-gami cells was a more efficient folding compartment for hGH than the periplasm (19). This high-yielding expression facilitated efficient synthesis of hGH variants and enabled the cell proliferation–based experiments for dimeric GH-C53S.

Dimeric GH-C53S–His did not bind to hGHBP and induced no significant proliferation in the BaF/B03 assay. The residual bioactivity of high concentrations of GH-C53S dimer can be attributed to the presence of minimal amounts of monomeric hGH. In the GH-C53S dimer, the two binding sites 1 are linked closely by a disulfide bridge between the two p.Cys-165 amino acid residues and are thus not accessible to GHR interaction. Because of the short disulfide bridge closely linking the two binding sites 1, such dimeric hGH molecules retain only two binding sites 2 for receptor interaction. Our data lend further support to the assumption that binding site 2 alone is incapable of binding to the hGH receptor (20). Cunningham et al. (20), in their seminal article, saw no evidence of formation of a hGH–hGHBP complex mediated exclusively through site 2. Beyond the lower affinity, it is reasonable to speculate that a dimer with two symmetrical binding sites 2 will not permit rotation of the receptor subunits, unlocking JAK2 transphosphorylation (21, 22).

Cytoplasmic proteins do not generally contain structural disulfide bonds, as the latter are posttranslational modifications requiring translocation to the ER (23), which is where their native disulfide bonds are formed, which is typically essential for the stability of the protein (24). The secretory pathway of the AtT20 cells resembles somatotrophs, which differs from other nonendocrine cells (25). To form the dense cores of secretory granules, the first step is packaging through aggregation,
involving high concentrations of Zn\(^{2+}\) ions (26, 27). These Zn\(^{2+}\) ions have been reported to induce transient formation of hGH dimers (28). In addition, in vitro experiments revealed the existence of self-associated intermediates in the hGH folding pathway and a concentration-dependent association, including noncovalently linked dimer formation, during folding (29, 30). Furthermore, the acidic milieu in secretory vesicles (pH 5.5) promotes formation of disulfide bonds (31), and genes encoding proteins with protein disulfide isomerase activity are enriched in pituitary cells (32). These factors together are likely to increase aberrant disulfide bond formation for GH-C53S dimers in pituitary cells but not in other mammalian cells. We also reproduced formation of this dimer in GC cells, a rat pituitary cell line derived from the GH3 strain (33). In contrast, dimer formation of GH-C53S in CHO-K1 and HEK-293 cells was not significantly elevated compared with GH-WT.

Without stimulation, GH-C53S was secreted at a significantly higher rate in pituitary AtT-20 cells compared with GH-WT. However, unlike GH-WT, secretion of GH-C53S and GH-C53S/C165A could not be stimulated using a secretagogue. The immunofluorescence images revealed that GH-C53S and GH-C53S/C165A were only sparsely processed into granules and instead remained in the Golgi apparatus, as evident by coexpression of hGH and β-COP, a protein associated with the Golgi apparatus and non-clathrin-coated vesicles (35). Correspondingly, lysates of GH-C53S–transfected cells contained significantly less GH compared with GH-WT. The overall expression pattern was very similar to hGH mutants lacking the C-terminal disulfide bond (36). Unlike GH-del32–71 (exon three deletion), which induces ER stress and apoptosis (37), GH-C53S is not retained in the ER but in the Golgi apparatus. Taken together, the mutation p.C53S in GH abolishes cells’ ability to release GH upon a stimulus and abolishes GH granule formation. However, basal secretion is retained.

GH-C53S is partially O-glycosylated, as shown by the deglycosylation experiment in Fig. 4. The glycosylated pituitary-derived 24-kDa hGH isoform, representing a small amount of the total hGH generated in the pituitary (38), is O-glycosylated at threonine 60, which is located in the random coil sequence linking helix 1 and 2 (39). Disrupting the long disulfide bond in GH-C53S probably leads to enhanced mobility of the random coil sequence, which makes glycosylation of Thr-60 in this variant more feasible compared with GH-WT and causes a bigger proportion of GH-C53S to be glycosylated.

Disulfide bridges in proteins are important for protein stability (40). Monomeric GH-C53S–His had lower resistance to trypsin hydrolysis compared with GH-WT–His. Dimeric GH-C53S–His, however, displayed higher stability than GH-WT–His. This is in keeping with in vivo measurements, where 45-kDa hGH and dimers of 20-kDa hGH were cleared and degraded at a lower rate than monomeric hGH. Therefore, the ratio of dimeric/monomeric hGH in the blood is increased compared with the relative abundance of secreted forms (41), which would lead to a high proportion of bioinactive GH-C53S dimers compared with GH-C53S monomers with only reduced bioactivity.

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| primer         | sequence                                      |
|----------------|-----------------------------------------------|
| wt-fw          | 5′-CACCATGGCTACAGGCTCCCG-3′                   |
| C53S-rv        | 5′-GACCTTGAAGAAGAGGGAGGT-3′                   |
| C53S-fw        | 5′-CAGACCTCCCTCTCTCTCTCAGA-3′                 |
| wt-rv          | 5′-GAAGCCACAGTGCCCCACCC-3′                    |
| pET-101-his-fw | 5′-CAATAGCCCAACACTTCCCTATCCA                   |
| pET-101-his-rv | 5′-TTAATGTTGATGGTGATGTGGAGAC                    |

**Figure 11. List of primer sequences.**

In conclusion, our study reveals a new molecular mechanism causing bioinactive GH (Kowarski syndrome). Replacement of p.Cys-53 with serine in hGH causes formation of a bioinactive hGH dimer from two bioactive, monomeric, mutant GH-C53S molecules by an intermolecular disulfide bond. Additionally, the high-level cytoplasmic expression of hGH variants in *E. coli* described in this paper is an effective tool to generate recombinant hGH.

**Experimental procedures**

**Generation of the pcDNA3.1-hgh and pet101 vectors**

Mutant hGH cDNA was generated using site-directed mutagenesis of hGH cDNA or previously generated hGH-C165A cDNA as a template with Pfu polymerase with Pfu buffer (Promega) or Phusion polymerase with 5× Phusion HF buffer (New England Biolabs) and dNTP mixture. The chosen primer sequences are shown in Fig. 11. Plasmids were purified using agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The PCR products containing the signal sequence were cloned in the pcDNA™3.1D/V5-His-TOPO® vector using pcDNA™3.1 Directional TOPO® Expression Kit, and products without the signal sequence but with a His\(_{6}\) tag were cloned in the pET101/D-TOPO® vector using the Champion™ pET Directional TOPO® Expression Kit (all vectors from Thermo Fisher Scientific). The vectors were transferred in TOP10® *E. coli* (Thermo Fisher Scientific), and the cells were streaked on agar plates containing carbenicillin and incubated at 37 °C for 16 h. Selected colonies were incubated o.n. at 37 °C in 5 ml of Terrific Broth medium (Carl Roth, Karlsruhe, Germany). Mutagenesis was confirmed by restriction digestion with PstI followed by sequencing (Eurofins MWG Operon, Ebersberg, Germany; pCR3.1-BGHrev, T7, and T7term as primer). Cells carrying the correct plasmid sequence were inoculated in 100 ml of Terrific Broth medium (Carl Roth) and incubated o.n. with 37 °C, and plasmids were purified using the Plasmid Midi Kit (Qiagen).

**Cell culture and treatment**

All cell cultures were kept in a humidified atmosphere with 10% CO\(_{2}\), 50 units/ml penicillin/streptomycin, 1 mM pyruvate, and 4 mM L-glutamine. AtT20 (ATCC, catalog no. CCL-89, RRID: CVCL_2300), GC, HEK-293 (RRID: CVCL_0045), and CHO-K1 cells were grown in DMEM supplemented with 10% fetal bovine serum. BaF/B03 and Nb2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.
**Production of GH variants in mammalian cell lines**

HEK293, CHO-K1, AtT20, and GC cells were transfected with 0.8 μg of pcDNA3.1-hGH plasmids in 98 μl of Opti-MEM in the presence of 2 μg of Lipofectamine 2000 (Thermo Fisher Scientific). 72 h after transfection, the supernatants were harvested. Lysates were made using 200 μl of lysis buffer per well (20 mM Trizma base, 1.7 mM NaCl, 2.5 mM EDTA, 10% glycerol, 1% Triton X-100 (Sigma-Aldrich), and Complete Mini Protease Inhibitor® (Roche, Basel, Switzerland)). Cell debris was cleared with centrifugation for 5 min at 10,000 × g, and the supernatants were stored at −20 °C. For proliferation assays, 24 h after transfection of CHO-K1 cells, the medium was changed to serum-free ISF-1 medium (Biochrom, Berlin, Germany). To control for differences in transfection efficiency, we cotransfected AtT-20 cells with hGH variants and equal amounts of PlGF, a 46- to 50-kDa homodimeric glycoprotein, and saw no effect on PlGF synthesis.

**Stimulating hGH secretion from transfected AtT20 cells**

Transient transfection was performed as described above. After 12 and 14 h, the culture medium was changed to 200 μl of normal culture medium or medium supplemented with 5 mM 8-Br-cAMP (Sigma-Aldrich) per well. Supernatants were collected after 12, 14, and 16 h, and 200 μl of lysates per well were generated after 16 h as described above. hGH content in supernatants and lysates was measured using the GH assay as described below.

**Generation of the GH-C53S dimer in E. coli**

To overcome low expression levels of GH-C53S in pituitary cells, we also chose to express this mutant in *E. coli*. Periplasmic expression in BL21 Star cells with a pET vector employing an ompA leader sequence yielded little dimeric hGH and low amounts of GH-WT. In contrast, cytoplasmic expression in Rosetta-gami B DE3 cells (Merck Millipore) yielded high amounts of dimeric GH-C53S (about 0.1 mg/1 liter of culture) and GH-WT (about 0.5 mg/1 liter of culture). We transformed Rosetta-gami B DE3 cells with the pET101 vector containing the protocol developed by Sockolosky and Szoka (19). Expression closely followed the protocol developed by Sockolosky and Szoka (42) for periplasmic expression. Cell cultures were centrifuged, and the cell pellet was resuspended in 10 ml of Cell Lytic B (Sigma-Aldrich) per gram of wet cell paste and incubated for 10 min at 4 °C. After 10-min centrifugation at 8000 × g, the His6-tagged protein was extracted from the supernatant using nickel-nitrotetraacetic acid–agarose (Thermo Fisher Scientific) in disposable columns following standard procedures (PBS (pH 7.4); equilibration buffer, 10 mM imidazole; wash buffer, 30 mM imidazole; elution buffer, 300 mM imidazole). The eluates were centrifuged and filtered through 0.45-μm filters, and the pH was adjusted to 7.4. hGH was further purified by affinity chromatography with the anti-hGH monoclonal 10A7 antibody (43) coupled to a HiTrap NHS activated HP column (GE Healthcare, Little Chalfont, UK). hGH was eluted with 0.1 M glycine (pH 2.0) elution buffer. Western blotting confirmed the presence of ~75% dimeric hGH in the eluates. The eluates were supplemented with 10% glycerol and further concentrated using VivaSpin 500 5000 MWCO columns (Sartorius, Göttingen, Germany). Size-exclusion chromatography with two connected columns (Superdex 75® followed by Superdex 200 GL®, GE Healthcare) separated the monomer from the dimer (flow rate, 0.5 ml/min; fraction size, 0.25 ml). The purity of generated hGH variants was assessed in Coomassie- and silver-stained SDS gels and generally exceeded 50%. The amount of dimeric and monomeric hGH generated was calculated in quantitative Western blots.

**Western blotting**

Cell lysates and supernatants were heated to 95 °C for 5 min and loaded with 5× lane marker nonreducing/reducing sample buffer (Lane Marker Sample Buffer, Thermo Fisher Scientific) on a 5% acrylamide stacking and 12% resolving SDS gel. HEK and CHO samples were concentrated 10-fold using immunoprecipitation, employing the primary antibodies described below. Nitrocellulose membranes (Hybond ECL, GE Healthcare) were blocked o.n. with 5% milk powder. Primary hGH-antibodies were the in-house-generated monoclonal antibodies 7B11 and 10A7 (1 μg/ml each, see Ref. 43 for antibody details). The secondary antibody was HRP-linked anti-mouse IgG (GE Healthcare, NA 931, RRID: AB_772210). All antibody solutions contained 1% BSA and 0.5% milk powder. Blots were treated with ECL detection reagent, exposed to Amersham Biosciences Hyperfilm ECL films (GE Healthcare), and developed with an Agfa Curix 60 processor or exposed to ECL Select detection reagent, where bands were detected with a Typhoon 8600 laser scanner (Amersham Biosciences).

**Glycosylation of GH variants**

9 μl of supernatants from GH-WT and GH-C53S–transfected CHO-K1 cells and 1 μl of 10× glycoprotein denaturing buffer were incubated at 100 °C for denaturation, followed by addition of 2 μl of 10% NP40, 2 μl of 10× G7 reaction buffer, 2 μl of neuraminidase (50,000 units/ml), and 5 μl of O-glycosidase (40,000,000 units/ml) (all deglycosylation reagents from New England Biolabs) and incubation at 37 °C for 1 h. Samples were subjected to Western blotting as described above.

**GH assay**

The concentration of hGH was measured by immunoassay with the in-house antibodies 7B11 and 10A7 (43, 44). The assay is designed to assess all hGH variants equally. mAb 10A7 binds hGH at a distance from both receptor interaction sites, and mAb 7B11 binds to site II of the GHR, which is not affected by the C53S mutation. However, because of formation of GH-C53S dimers in pituitary cells, we performed multiple quantitative Western blots on GH-C53S generated in AtT-20 and GC cells and found underreporting of GH-C53S compared with GH-WT in pituitary cell supernatants and lysates. We therefore corrected the values generated for GH-C53S in pituitary cells by the immunoassay with a factor of 4.3.

**Receptor binding assay**

The affinity of hGH generated in Rosetta-gami B DE3 cells and purified as described was compared with pituitary hGH in...
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an assay where increasing amounts of hGH competed with biotinylated rhGH (5 ng/well) in binding to hGHBP (PLR Ltd., 5 ng/well), captured by 10B8 antibody (45) (500 ng/well). Bound rhGH-b was detected by incubation with europium-labeled streptavidin; europium was detected with a Victor3 plate reader (PerkinElmer Life Sciences).

Proliferation assays

Bioassays were performed with BaF/B03 cells expressing full-length GHR (46) using His-tagged hGH produced in Rosetta-gami B DE3 cells. Deviating from the original protocol, we used 2.5 × 10^5 BaF/B03 cells in 100 μl/well and final hGH concentrations between 0.01 and 50 ng/ml to increase the assay’s sensitivity. Nb2 cells expressing rat PRLR (47) were also used, with 10^4 cells in 100 μl/well. To determine the number of viable cells, we used the Via Light Plus Kit (Lonza, Basel, Switzerland), measuring the amount of ATP. Before exposure of the cells to hGH, they were starved overnight in RPMI medium with 0.5% horse serum and no FBS.

Trypsin digestion

Serum-free CHO-K1 supernatants and hGH generated in E. coli were diluted to a final concentration of 500 ng/ml hGH. 10 μl of 4 μg/ml trypsin in 1 mM HCl was added to a first fraction (100 μl), and 10 μl of 1 mM HCl was added to a second fraction (100 μl). The samples were incubated at 37 °C with gentle agitation for 2 h. After different time periods over 120 min, 16-μl samples were taken from the fractions and stored at −20 °C, until reanalysis by Western blotting.

Immunofluorescence staining

AtT-20 cells were grown on glass coverslips in 12-well plates and transfected with hGH mutants as described above. Staining was performed following the Protocol for the Preparation and Fluorescent ICC Staining of Cells on Coverslips developed by R&D Systems. Primary antibodies used were 10 μg/ml of in-house murine 10A7 against hGH and 20 μg/ml of rabbit coatomer subunit β (β-COP, a Golgi marker protein) antibody (Thermo Fisher, PA1-061, RRID: AB_2081296). Secondary antibodies were 1:50 anti-mouse IgG fluorescein-linked whole antibodies (GE Healthcare, N1031, RRID: AB_1062594) and 1:10 Cy3 AfinityPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 711-165-152, RRID: AB_2307443).

Statistics

Values are reported as mean ± S.D. For standard deviations and p values, nonlinear regression, and IC_50/EC_50 calculations, Prin 6.0 (GraphPad) was used. Standard deviations and p values were calculated using one- or two-way ANOVA with Bonferroni’s or Tukey’s multiple comparisons test.

Data availability

All data discussed in this paper are contained in this paper. A quantitative immunofluorescence analysis related to Fig. 4 is available in Fig. S1.

Author contributions—M. S., Z. W., and C. J. S. conceptualization; M. S. and Z. W. data curation; M. S. formal analysis; M. S., Z. W., and C. J. S. validation; M. S. and Z. W. investigation; M. S. visualization; M. S. methodology; M. S. writing-original draft; Z. W. and C. J. S. resources; Z. W. and C. J. S. supervision; Z. W. and C. J. S. funding acquisition; Z. W. and C. J. S. project administration; Z. W. and C. J. S. writing-review and editing.

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