Aus der Klinik für Endokrinologie und Stoffwechselmedizin (einschl. Arbeitsbereich Lipidstoffwechsel)
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DISSERTATION

Die molekularen Mechanismen des angeborenen Wachstumshormonmangels verursacht durch Cystein-Mutationen

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Inhaltsverzeichnis

Tabellen und Abbildungsverzeichnis

Abstrakt: Die molekularen Mechanismen des angeborenen Wachstumshormonmangels verursacht durch Cystein-Mutationen

Hintergrund: menschliches Wachstumshormon (hGH) enthält zwei Disulfidbrücken, die auch in anderen Spezies und in Prolaktin vorliegen. Es ist bekannt, dass hGH mit reduzierten und alkylierten Cysteinen, dadurch frei von Disulfidbrücken, fast die gleiche Bioaktivität wie Wildtyp hGH in Tiermodellen hat. Für diese Arbeit identifizierten wir Patienten mit angeborenem Wachstumshormonmangel und Mutationen der Cysteine im Wachstumshormonmolekül. Dann führten wir verschiedene Untersuchungen durch, um den jeweils zugrundeliegenden Mechanismus des Wachstumshormonmangels festzustellen. Zusätzlich untersuchten wir die Eigenschaften von Wachstumshormon ohne die Disulfidbrücken, ohne diese chemisch zu zerstören.

Methoden: Plasmide mit hGH-cDNA, die der cDNA der betroffenen Individuen entspricht wurden mittels site-directed mutagenesis erzeugt. Um hGH frei von Cysteinen zu erzeugen ersetzten wir alle Cystein-codierenden Tripletts mit Alanin-codierenden Tripletts. Das mutierte Wachstumshormon wurde dann in Hypophysenzelllinien (AtT-20 und GC) exprimiert und analysiert mit Immunohistologie, Immunoassays und Western-Blots. hGH und hGH-Varianten exprimiert in CHO-K1 und HEK Zellen wurden aufgereinigt und die Bioaktivität in Zellproliferationsassays mit stabil mit dem Wachstumshormonrezeptor transfizierten Baf/B03 und einen trunkierten Prolaktinrezeptor exprimierenden Nb2 Zellen bestimmt. Rezeptorbindung der Mutanten wurde in Kompetitionsassays mit GHBP (growth-hormone binding protein) untersucht. Um die Stabilität der Mutanten zu untersuchen wurde die Resistenz gegen Trypsinverdau untersucht.

Ergebnisse: Unsere umfangreiche Analyse identifizierte eine Vielzahl von Mechanismen, welche den Funktionsverlust durch die Cysteinmutationen bedingen. Manche hGH-Mutationen zeigten eine stark reduzierte Expression und Sekretion in Hypophysenzellen, andere hatten eine signifikant reduzierte Affinität zu dem Wachstumshormonrezeptor und eine reduzierte Bioaktivität. Bemerkenswerterweise bildet die hGH-C53S Variante disulfid-verbundene Dimere, die den Wachstumshormonrezeptor nicht binden oder aktivieren können. HGH ohne Disulfidbrücken hatte eine nur gering reduzierte Bioaktivität, allerdings eine deutliche verringerte Resistenz gegenüber Trypsinverdau.

Schlussfolgerungen: Die Disulfidbrücken im Wachstumshormon sind nicht essentiell für die Bindung und Aktivierung des Wachstumshormonrezeptors, da die Bioaktivität ohne Disulfidbrücken nur gering reduziert ist. Sie sind allerdings entscheidend für die strukturelle Stabilisierung des Moleküls zum Schutz vor proteolytischen Abbau, was essentiell für die Aufrechterhaltung von ausreichenden hGH-Spiegeln in der Zirkulation im Organismus sein könnte. Die molekularen Mechanismen, die einen angeborenen Wachstumshormonmangel durch Cysteinmutationen im Wachstumshormon bedingen sind vielfältig und schließen die erstmals beschriebene Bildung eines bioinaktiven Wachstumshormondimers ein.

Abstract: The molecular mechanisms underlying Inherited Growth Hormone Deficiency caused by cysteine mutation

Background: HGH contains two disulfide bridges, which are highly conserved among species and are also found in prolactin. It is well established, that hGH with reduced and alkylated cysteines, therefore devoid of disulfide bonds, has almost full wildtype bioactivity in animal models. For this study, we identified published cases of patients with Inherited Growth Hormone Deficiency and mutations of the cysteines in the hGH-molecule. We then conducted a number of studies to identify the underlying mechanisms. Additionally, we investigated the properties of hGH lacking all disulfide bonds, without disrupting them chemically.

Methods: Plasmids containing hGH-cDNA matching the cDNA of the affected individuals were generated using site-directed mutagenesis. To generate hGH devoid of all disulfide bonds, we replaced all triplets encoding cysteine with triplets encoding alanine. Mutant growth hormone was then expressed in pituitary AtT20 as well as GC cells and analysed by immunohistology, immunoassay and western-blotting. hGH and hGH-variants generated in CHO-K1 and HEK cells were purified and used to examine their bioactivity in Baf-B03 cells stably expressing the GHR and Nb2 cells expressing a truncated prolactin receptor. Receptor binding of the mutants was studied in competition assays with GHBP (growth-hormone binding protein). To determine the stability of the mutants they were subjected to trypsin digestion.

Results: Our extensive analysis revealed a variety of mechanisms which are responsible for the loss of biological function of the hGH variants. Some hGH-variants showed greatly reduced expression and secretion in pituitary cells, others showed significantly reduced affinity to the GHR and a reduction in bioactivity. Most strikingly, the hGH-C53S variant forms disulfide-linked dimers which are unable to bind and activate the GHR. HGH completely devoid of disulfide-bonds only had slightly diminished bioactivity compared to hGH-wt, however resistance to trypsin digestion was greatly reduced.

Conclusions: The disulfide bonds in hGH are not essential to for binding to- and activate activation of the GHR, as the bioactivity of cysteine-free hGH is only moderately reduced. However, they these are structurally stabilizing the hormone by rescue from proteolytic cleavage, which might be essential for the maintainance of circulating hGH levels in vivo. The molecular mechanisms underlying Inherited Growth Hormone Deficiency caused by cysteine mutation are diverse and include the newly discovered formation of a bioinactive hGH-dimer.

Manteltext:

Introduction:

The physiology of Human Growth Hormone

Human growth hormone is a hormone with a variety of biological functions as the growth hormone receptor is expressed in almost all tissues (2). hGH`s main physiological role is the promotion of growth in childhood and adolescence, but it also plays a vital role in adults. hGH ubiquitously induces cell proliferation and differentiation, both directly through the growth hormone receptor and indirectly through IGF-1 (3).

Growth Hormone Deficiency

Growth hormone deficiency is characterized by different features, depending on the age of onset in the affected individual. In childhood and adolescence, its most notable feature is growth failure, ultimately leading to short stature (defined as height 2 SD below the population mean). The assessment of the patient`s auxology is essential to diagnose GHD in children (4). Additionally, increased weight/height ratio, delayed bone age and an immature face are potential signs of growth hormone deficiency in children. Before the definitive diagnosis of GHD in children can be made, a variety of other conditions causing growth failure has to be considered (5). Biochemical assessment involves measurement of hGH and IGF-I using immunoassays. Short stature and IGF-1 levels below 2 SD of the reference range standardised for sex and age strongly suggest GHD. Because of the pulsatile nature of hGH release, hGH levels are more difficult to interpret, however a peak GH concentration below 10 µg/L has traditionally been used to support the diagnosis. In some cases, a GH provocation test can provide further evidence to support the diagnosis of GHD; substances to induce GH secretion include arginine, clonidine, glucagon, insulin and l-dopa. These tests should only be conducted and interpreted by experienced paediatric endocrinologists (5).

In adults, clinical features include an altered body composition with an increase in fat mass, abdominal adiposity, reduced bone mineral density, reduced muscle mass and limited physical performance (6). Metabolic alterations, such as increased total cholesterol and decreased basal metabolic rate occur. There is growing evidence, that hGH is important to maintain normal heart function and GHD deficient adults have an increased risk of cardiovascular disease (7). Furthermore, adults with growth hormone deficiency report and decreased psychological well-being with disturbances in all areas of private life (8).

A special group of patients is affected from birth by the absence of the biological function of hGH, often caused by Inherited Growth Hormone Deficiency (IGHD). This is a rare condition with a complex phenotype characterized by being small for the gestational age, severe growth retardation and delayed bone age. Additionally, prolonged hypoglycaemia can be found in children with IGHD, particularly, if the corticotrope axis is also impaired (9). The guidelines advise evaluation of a genetic disorder in the following circumstances: 1) early onset of growth failure, 2) positive family history and possible consanguinity, 3) height more than 3 SD below the mean, and 4) extremely low GH response to provocation tests, including GHRH, and very low IGF-I and IGF-binding protein-3 (IGFBP-3) levels (5). Genetically, there are different causes of IGHD, which the most common being mutations in the GH1 gene, encoding hGH (10).

The structure of GH1 and hGH

GH1 is located on the long arm of chromosome 17 (17q22–24) together with four related genes with a high degree of sequence homology arisen through gene duplication. The pituitary-specific expression of GH is regulated by a highly polymorphic proximal promoter and a locus control region 15 to 32kb

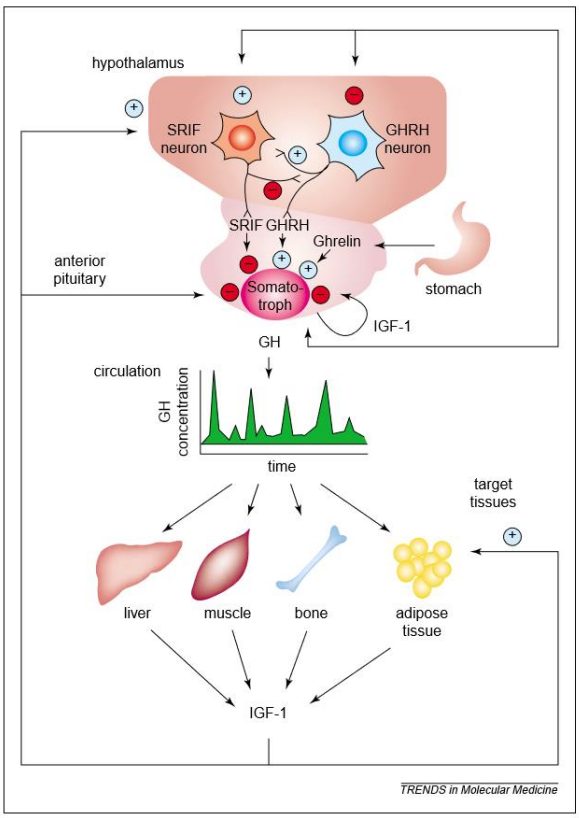
upstream of GH1 (11). Several isoforms of hGH are generated from GH1 trough mRNA splicing and posttranslational modifications (12).

The main product is a 22 kDa, 191 amino acid, single chain protein. Alternative splicing of exon 3 generates 20 kDa hGH, devoid of amino acids 32-46, which makes up 5-10% of total hGH (13). Additionally, oligomers of hGH have been described, most prominently a 44 kDa hGH dimer (14).

The hGH molecule is a proteohormone, consisting of four helices in up-up-down-down configuration. A long crossover connection, consisting of residues 35 to 71, links helix 1 to helix 2, and a similar connection (residues 129 to 154) is found between helices 3 and 4. The first connection is disulfide-bonded to helix 4 through p.Cys53 and p.Cys165. A second disulfide bond connects the COOH-terminus to helix 4 with a disulfide bond between p.Cys182 and p.Cys189 (15). These four cysteines (p.Cys53, p.Cys165, p.Cys182, p.Cys189) and the two corresponding disulphide bonds are highly conserved among species. They are also found in the structurally related hormone Prolactin, a hormone which plays a crucial role initiation of lactation in females. The high degree of conservation of structural features of proteins in general indicates a functional significance. For hGH however, there is a number of studies which provided ample evidence, that the disulphide bridges are not essential for biological function (16-19).

The growth hormone axis

To understand, how a hormone`s function can be affected, one has to understand its way of action. This scheme illustrates the endocrine regulation of hGH.



Scheme from: Okada and Kopchick (1).

As illustrated, GHRH is generated in the hypothalamus and stimulates the production and pulsatile secretion of hGH from somatotrophs in the pituitary. Somatostatin (somatotropin release–inhibiting factor, SRIF) inhibits the release of GHRH and hGH. Additionally, there are a number of hormones from peripheral organs that stimulate hGH release.

Circulating hGH binds to the GHR and stimulates the release of IGF-1, which acts on the target tissues. In a classical endocrine feedback loop, IGF-1 levels suppress the release of hGH and GHRH and stimulates Somatostatin. Thus, the release of hGH in the neuroendocrine anterior pituitary is pulsatile. In circulation, hGH is mainly bound to a specific growth hormone binding protein (GHBP), which structurally equals the GHR`s extracellular part.

Table 1

On a cellular level, the release of hGH from somatotrophs is a highly complex and tightly regulated process (20). Briefly, hGH is synthesised in the ER, transported to the golgi apparatus and through a process involving aggregation of hGH molecules and accumulation of membrane proteins dense-core granules are generated (21). These are membrane enclosed vesicles containing tightly packed hGH to

enable rapid release form the somatotrophs upon stimulation through fusion of the vesicles with porosomes in the cell membrane of somatotrophs (20).

Growth hormone receptor activation

The GHR is a classical class 1 hematopoietic cytokine receptor which exists as a constitutive dimer in the cell membrane. hGH contains two binding sites, the high affinity site 1 and the low affinity site 2, which are asymmetrically placed (15). Hormone binding to the first subunit of the GHR initiates activation and binding of site 2 realigns the subunits by rotation and closer apposition, resulting in juxtaposition of the catalytic domains of the associated tyrosine-protein kinase JAK2 below the cell membrane. This change results in activation of JAK2 by transphosphorylation, then phosphorylation of receptor tyrosines in the cytoplasmic domain, which enables binding of adaptor proteins, as well as direct phosphorylation of target proteins, notably STAT5 (22). In addition, there is activation of Src-family kinase, which activates the Ras/ERK-pathway. Activation of this kinase requires a slightly different conformational change in the GHR than needed for JAK2 activation (23).

Aim of this study

Through literature studies, we identified a number of patients with and without IGHD, in which mutations in hGH affecting the disulphide bridges were found. The finding, that growth hormone deficiency can be caused by mutations in GH1 affecting hGH`s disulphide bridges is surprising, considering the evidence, that hGH is not dependent on the disulphide bridges for its biological function. Key clinical and biochemical features, if available based on the published reports, of these patients are shown in the table below:

Mutant	GHD	S	HA	H	WT	GH-Peak	BA	SL	IGF-1	Published in
p.C53S	y	M	9	-3,6		44,7	r	n	low	(24)
p.C182X	y	F	8,12	-5,70	-3,7	0,16			<3,5 SDS	(25)
p.R77C	y	M M	5,6 20	-6,1 -1,9		38 253	r / n, r GHBP	n	low subnormal	(26)
p.S108C	y						n	r		(27)
p.R16C	n						n	r		(27)
p.F176C	n						r	n		(28)
p.C189R	n						n	n		(28)

Table 2

GHD: growth hormone deficiency, y: yes, n: no, S: sex, HA: age at height SDS, H: height SDS, WT: body weight, GH-Peak: in ng/ml, after stimulation, BA: biological activity, n=normal, r= reduced, SL: serum levels, n=normal, r=reduced

This work focuses on the mechanisms, by which mutations in GH1 affecting the cysteines in hGH are responsible for the phenotype of IGHD. We also assessed the biological properties of hGH devoid of all cysteines (and therefore disulphide bridges) with up-to-date methods.

The following key factors are essential for hGH`s bioactivity:

- Efficient synthesis, storage and release from the dense core granules of somatotrophs
- Protection from rapid degradation through proteases in the circulation
- The ability to bind the GHR
- The ability to activate the GHR

To investigate, why the mutations in GH1 leading to alterations in the cysteine bridges cause IGHD, we employed a set of experimental methods to address all the points listed above. The methods are described in detail in the publication “Short stature explained by dimerization of human growth

hormone induced by a p.C53S point mutation, Journal of Biological Chemistry" which contains a key finding of the studies of this work.

In the following, the methods are described briefly to give an understanding of the following results.

Methods:

Vector generation

First, the mutant hGH`s cDNA was generated using site-directed mutagenesis. The cDNA with hGH`s signal sequence was incorporated into the pcDNA™3.1D/V5-His-TOPO® vector using pcDNA™3.1 Directional TOPO® Expression Kit and the products without the signal sequence but with a hexa histidine tag were cloned in the pET101/D-TOPO® vector using the Champion™ pET Directional TOPO® Expression Kit. The vectors were amplified and purified for further use.

Cell culture and expression of hGH variants

All cell cultures were kept in a humidified atmosphere with 10% CO2, 50 units/ml penicillin/streptomycin, 1 mM pyruvate and 4 mM l-glutamine. AtT20 (ATCC Cat 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 were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Production of GH-variants in mammalian cell lines-HEK293, CHO-K1, AtT20 and GC cells were transfected with pcDNA3.1-hGH plasmids in Opti-MEM media in the presence of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA). 72 h after transfection, the supernatants were harvested. Lysates were made using 200 µl lysis buffer per well and cOmplete Mini protease inhibitor® (Roche, Basel, Switzerland)). Cell debris was cleared with centrifugation for 5 min with 10000 g, the supernatants were stored at -20 °C. For proliferation assays, 24 h after transfection of CHO-K1 the medium was changed to serum-free ISF-1 medium (Biochrom, Berlin, Germany).

Synthesis, storage and release from somatotrophs

To study the hGH mutants in conditions close to in vivo, we expressed the mutants cDNA in AtT-20 cells. These are mouse corticotrophic cells, that are proven to express hGH in a physiological manner (29). After transfection, we harvested the cells supernatants and generated cell lysates. Then, we used a highly sensitive assay to measure the hGH content supernatents (representing secreted hGH) and cell lysate (representing stored hGH).

For wt-hGH, GH-C53S and GH-C53S-C165A, transfected AtT-20 cells were fixed and immunostained for hGH, beta-COP (a golgi marker protein) and the nuclei were stained with DAPI.

Western-Blots

Equal volumes of cell supernatants or lysates were loaded with 5x lane marker nonreducing or reducing sample buffer (Lane Marker Sample Buffer, Thermo Fisher Scientific, Waltham, USA) following standard procedures. The membranes where blocked overnight and immunostained using the in-house monoclonal antibodies 10A7 and 7B11 as primary antibodies (for antibody details see: (30)) and HRP-linked anti-mouse IgG as secondary antibody (GE Healthcare NA 931, RRID: AB_772210). Blots were treated with ECL detection reagent, exposed to Amersham Hyperfilm ECL films (GE Healthcare, Little Chalfont, UK) 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, Little Chalfont, UK).

GHBP-binding assay

The affinity of the hGH variants generated was compared to pituitary hGH in an assay, where increasing amounts of hGH compete with biotinylated rhGH in binding to hGHBP captured by

monoclonal 10B8 antibody (43). Bound rhGH-b was detected by incubation with europium labelled streptavidin, europium was detected with on a Victor3 plate reader (PerkinElmer, Waltham, USA).

Proliferation assays

Bioassays were performed with BaF/B03 cells expressing full-length GHR (44) and Nb2 cells expressing a rat prolactin receptor (45). The cells were stimulated with increasing amounts of hGH variants. To determine the number of viable cells after stimulation, we used the Via Light Plus kit (Lonza, Basel, Switzerland), measuring the amount of ATP.

Trypsin digestion assay

Serum free CHO-K1 supernatants and hGH generated in E. coli were diluted to equal concentrations calculated after measurement with immunoassays. 10 µl of 4 µg/ml Trypsin in 1 mM HCl were added to a first fraction (100 µl) and 10 µl 1 mM HCl were 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 minutes, 16 µl samples were taken from the fractions and stored at -20°C, until re analysis by western-blotting as described before.

Results:

Expression in AtT-20 cells:

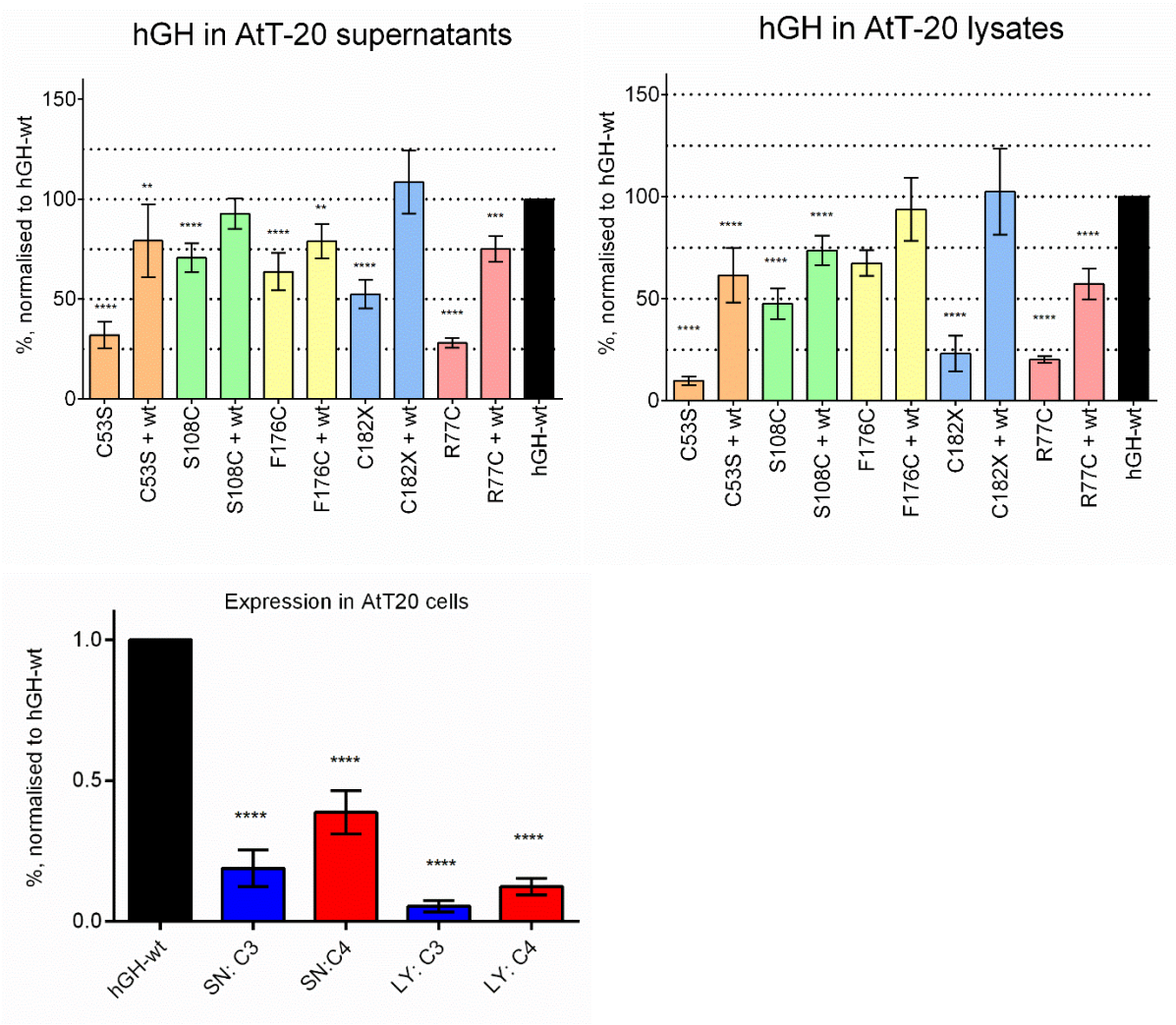


Figure 1. Expression of hGH in transfected AtT-20 cells. The results display the amount of hGH in supernatants or lysates normalised to hGH-wt levels (black column), the error bars represent the CI-

*95% of three experiments performed in duplicates. C3: hGH-C53S-C182A-C189A, C4: hGH-C53S-C165A-C182A-C189A. Statistics: One-way ANOVA with Bonferroni post-test comparing the means of each column with the control column (hGH-wt) * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.*

The results display a significant reduction of hGH in supernatants and lysates for almost all hGH-mutants compared to hGH-wt. Particularly the intracellular amounts were substantially diminished. Co-transfection of hGH-wt with the mutants in all cases did not result in a dominant negative effect on overall hGH expression. For hGH-C53S, hGH-R77C, hGH-C182X and the artificial hGH-C53S-C182A-C189A and hGH-C53S-C165A-C182A-C189A mutants, the diminished synthesis and secretion might impair the biological function of hGH in vivo.

GHR-Receptor binding assay

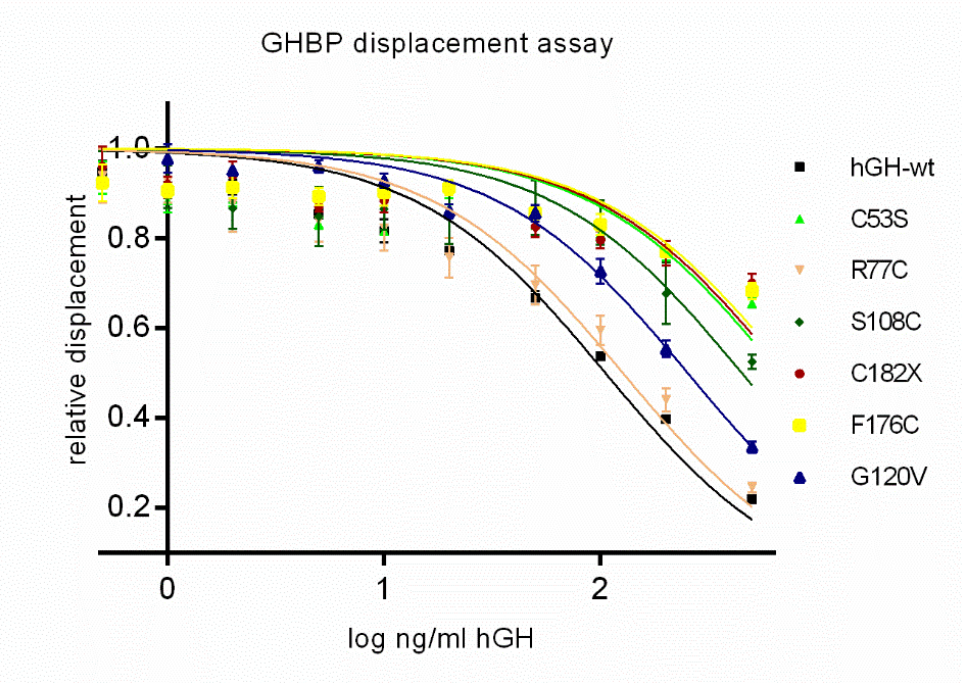


Figure 2 GHBP displacement assay. hGH variants synthesised in CHO-K1 cells displace rhGH-b (biotinylated recombinant human growth hormone) from binding to GHBP. The error bars display 95%-CI from three experiments performed in duplicates.

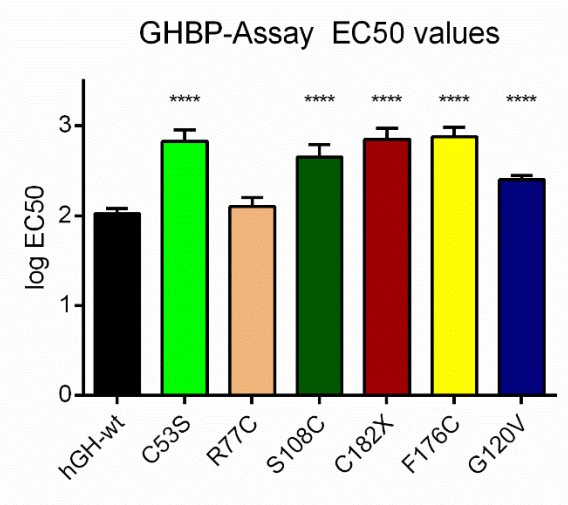
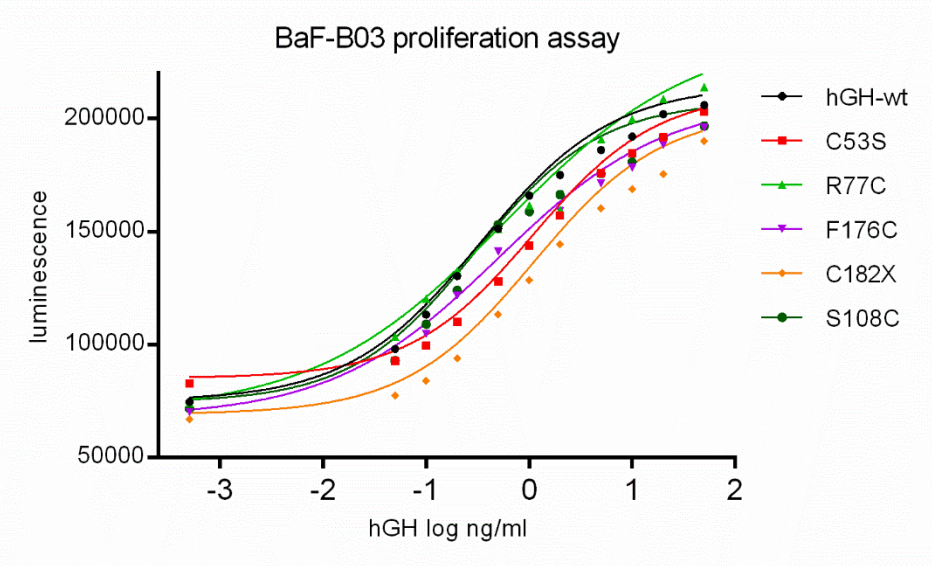


*Figure 3 GHBP displacement assay. EC50 values for hGH-mutants synthesized in CHO-K1 cells, calculated from the curves in Figure 2. Error bars display 95%-CI, data from three experiments performed in duplicates. Statistics: One-way ANOVA with Bonferroni post-test comparing the means of each column with the control column (hGH-wt) **** $P \leq 0.0001$.*

The affinities of hGH-C53S, hGH-S08C, hGH-C182X, hGH-F176C and hGH-G120V to the GHR are significantly reduced compared to hGH-wt, thus, significantly higher concentrations would be required to have the same biological effect of wt-hGH. hGH-R77C however has virtually the same affinity to the GHR as hGH-wt.

BaF-B03 proliferation assay



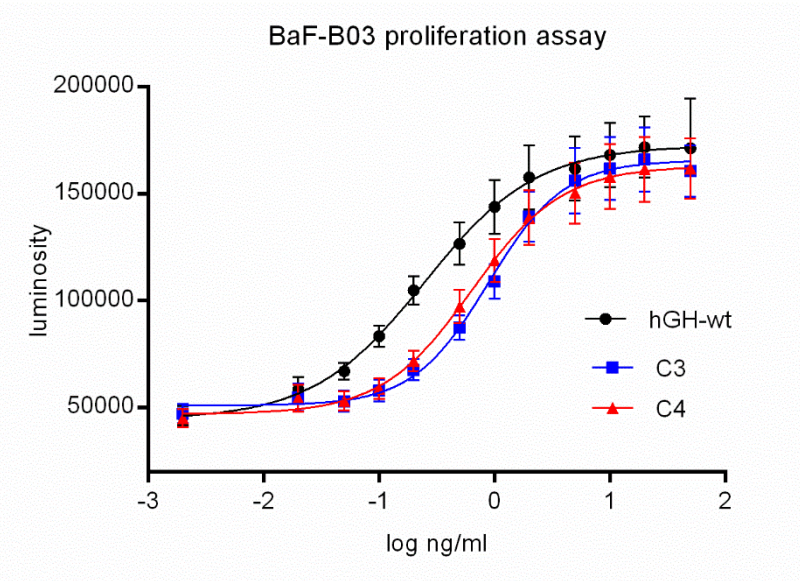
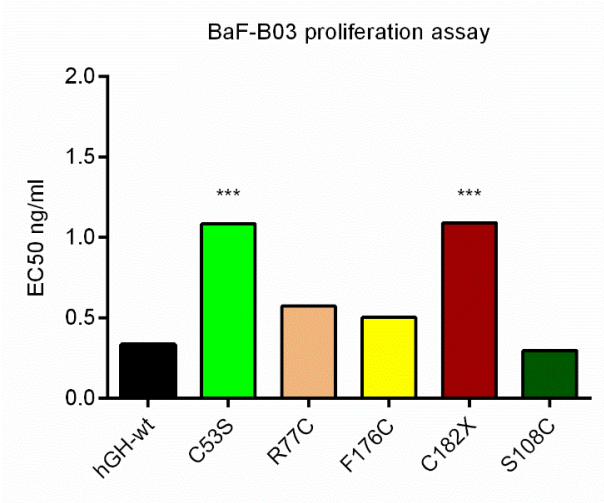


Figure 4 BaF-B03 proliferation assay. GHR-expressing BaF-B03 cells are stimulated with increasing amounts of hGH variants synthesised in CHO-K1 cells. C3: hGH-C53S-C182A-C189A, C4: hGH-C53S-C165A-C182A-C189A. The number of viable cells is measured via a luminesce assays measuring the amount of ATP. Error bars represent the SEM of three experiments performed in duplicates.



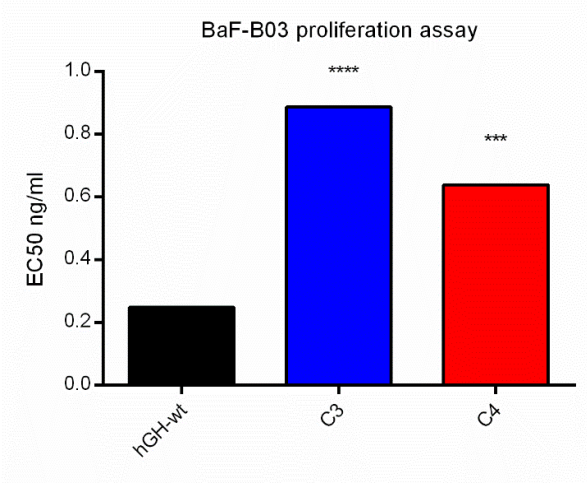


*Figure 5. BaF-B03 proliferation assay. EC50 values for hGH-mutants synthesized in CHO-K1 cells, calculated from the curves in Figure 4. C3: hGH-C53S-C182A-C189A, C4: hGH-C53S-C165A-C182A-C189A. No error bars as this is non-logarithmic. Statistics: One-way ANOVA with Bonferroni post-test comparing the means of each column with the control column (hGH-wt) *** $P \leq 0.001$, **** $P \leq 0.001$.*

The ability of hGH-C53S, hGH-C182X, hGH-C53S-C182A-C189A and hGH-C53S-C165A-C182A-C189A to bind and activate the GHR is significantly reduced compared to hGH-wt. In the cases of GHD in the patients with hGH-C53S and hGH-C182X a reduced bioactivity of the mutant hormone compared to hGH-wt is at least a contributing factor in the GHD. hGH-C53S-C182A-C189A and hGH-C53S-C165A-C182A-C189A confirm, that even if hGH is generated devoid of cysteines, its three-dimensional structure is still intact to allow activation of the GHR; although not as efficient as hGH-wt.

hGH-R77C, hGH-F176C and hGH-S108C do not differ from hGH-wt in its ability to bind and activate the GHR. In these patients, bioinactive hGH as a cause of GHD can be excluded.

Nb2 proliferation assay

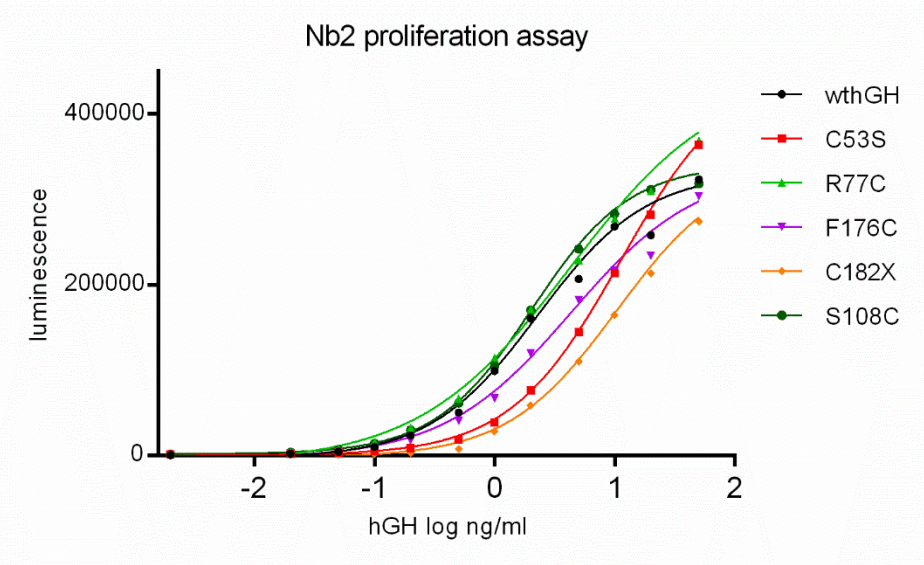


Figure 6. Nb2 proliferation assay. Nb2 cells expressing a truncated prolactin receptor are stimulated with increasing amounts of hGH variants synthesised in CHO-K1 cells. The number of viable cells is measured via a luminescence assays measuring the amounts of hGH variants synthesised in CHO-K1 cells. The number of viable cells is measured via a luminescence assays measuring the amount of ATP.

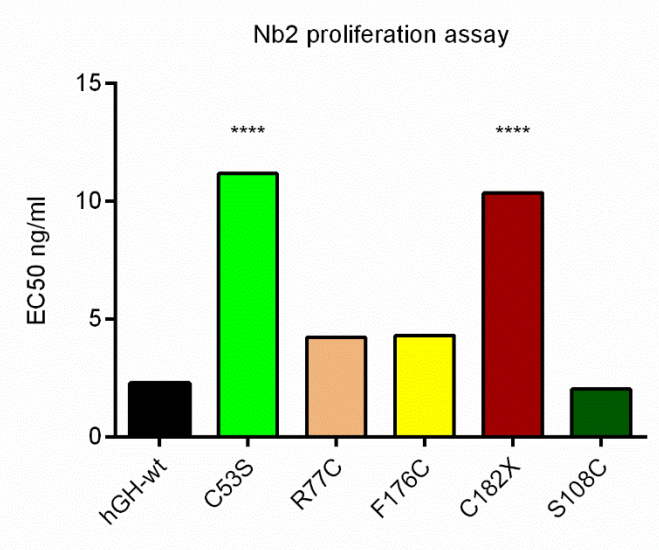


*Figure 7. Nb2 proliferation assay. EC50 values for hGH-mutants synthesized in CHO-K1 cells, calculated from the curves in Figure 6. No error bars as this is non-logarithmic. Statistics: One-way ANOVA with Bonferroni post-test comparing the means of each column with the control column (hGH-wt) **** $P \leq 0.0001$.*

hGH-C53S and hGH-C182X had significantly reduced ability to bind and activate the prolactin receptor. This matches the results from the BaF-B03 assay, where this mutant hGH variants also had reduced ability to bind and activate the GHR receptor. Overall, there seems to be no differential lactotropic or somatotropic activity for one of the mutant hGH variants in our study.

Western-blots:

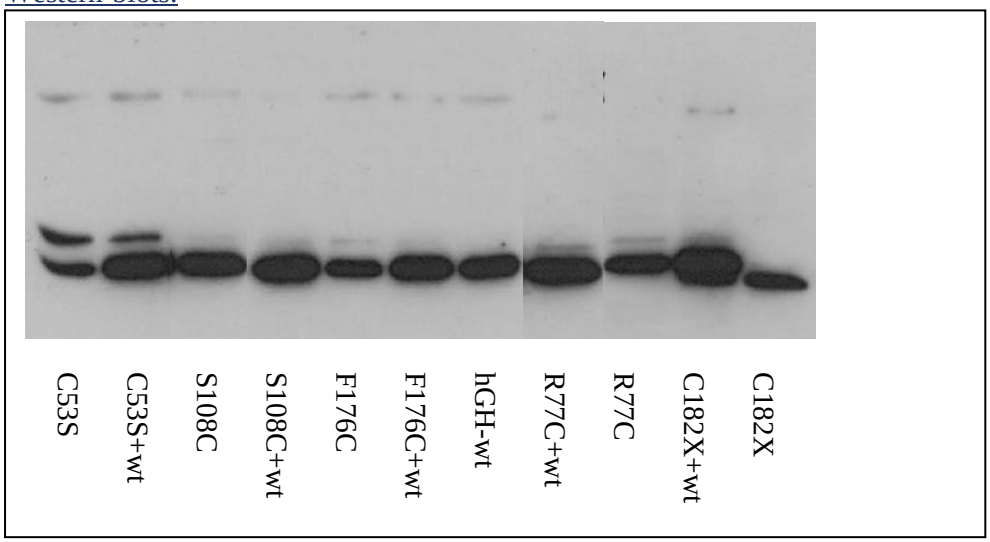


Figure 8. Western-Blots, reducing buffer, hGH staining with monoclonal Antibodies, equal amounts of transfected AtT-20 cell lysates. Spliced for presentation.

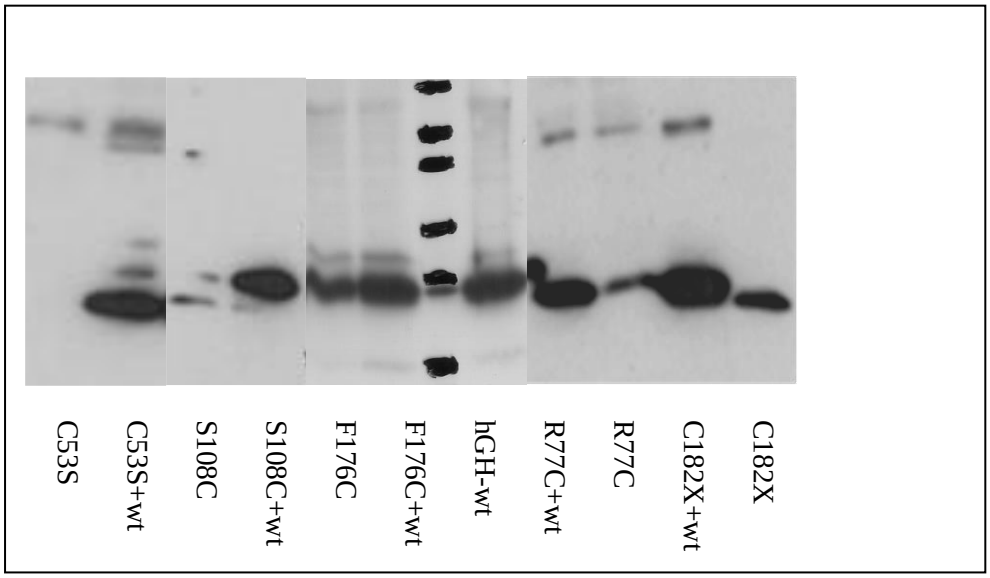


Figure 9. Western-Blots, non-reducing buffer, hGH staining with monoclonal Antibodies, equal amounts of transfected AtT-20 cell lysates. Spliced for presentation.

In the western-blots, we observed the presents of aberrant, 44 kDa hGH under non-reducing conditions for hGH-C53S and hGH-R77C expressed in AtT-20 cells. The 44 kDa hGH generally was not present under reducing conditions. The most likely explanation for this phenomenon is the formation of disulphide-linked hGH-dimers.

When the same mutant hGH variants where expressed in CHO-K1 or HEK-293 cells (mammalian, non-pituitary cell lines), no formation of aberrant hGH was observed (data not shown), which indicates that the hGH generation in specialised pituitatary cells, namely somatotrophs, differs substantially from other, non-pituitary cells. It highlights the importance of using an adequate expression system when generating proteohormones.

For the case of hGH-C53S, where this dimer is the dominating molecule in the cell lysates, we conducted a thorough analysis which can be found in the publication “Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation, Journal of Biological Chemistry”.

Trypsin digestion:

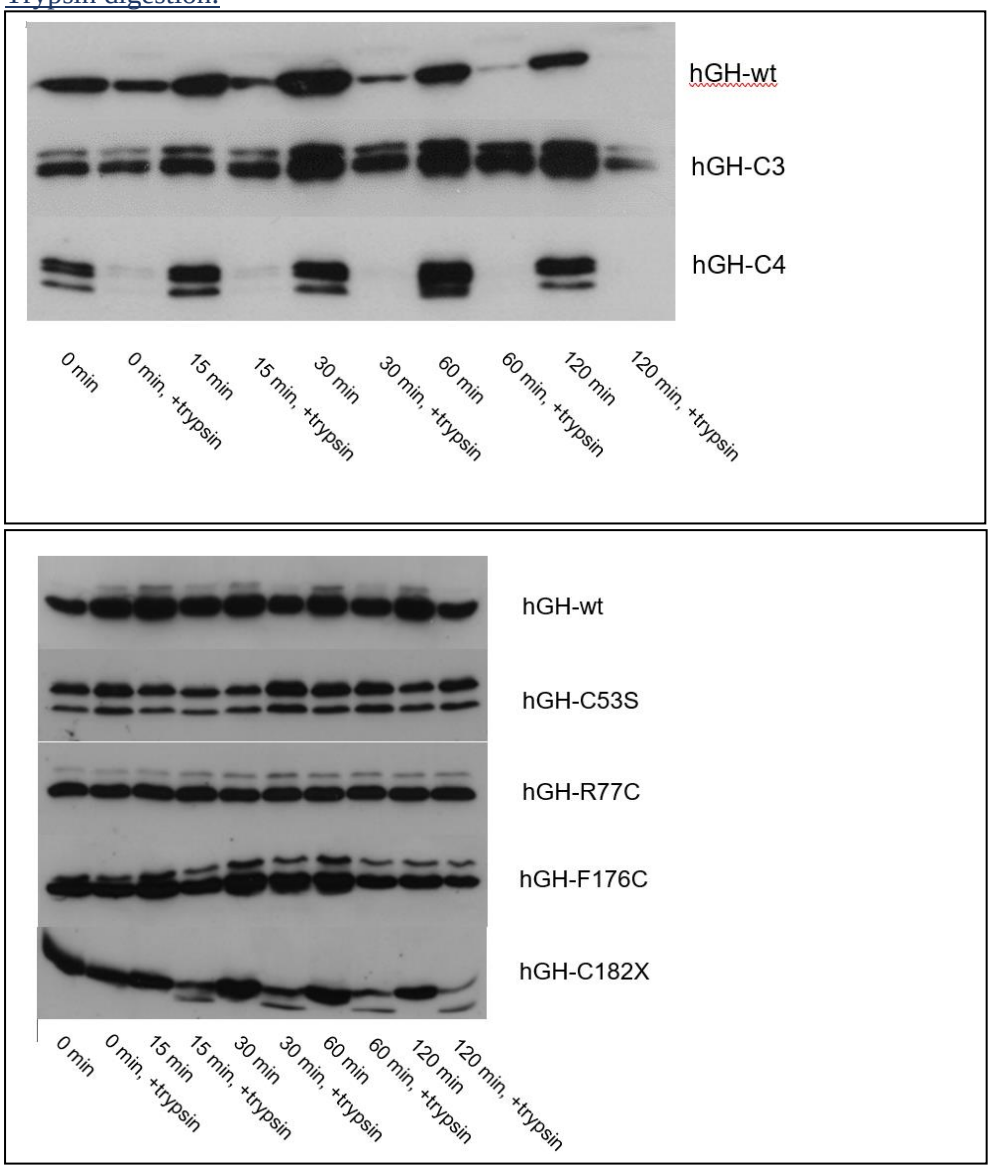


Figure 10. Western-Blots of trypsin-digested hGH, reducing buffer, hGH staining with monoclonal Antibodies 10A7 and 7B11. hGH-C3: hGH-C53S-C182A-C189A, hGH-C4: hGH-C53S-C165A-C182A-C189A

For hGH-C53S, hGH-R77C, hGH-F76C, no significant differences in the degradation compared to hGH-wt were observed. hGH-C182X however was digested at a significantly faster rate compared to hGH-wt, as evidenced by the weaker bands when exposed to trypsin. This highlights the importance of the c-terminal disulphide bridge for hGH`s stability.

hGH-C53S-C165A-C182A-C189A is significantly less resistant to trypsin digestion compared to hGH-wt as evidenced by the weaker bands in the present of trypsin throughout the experiment. This

indicates a major role of the disulphide-bridges in hGH for maintaining the proteins integrity in vivo. Presumably, the three-dimensional folding of hGH devoid of disulphide-bridges is still intact to allow activation of the GHR, however the molecule is not compact enough to avoid being rapidly degraded by proteases. In one sentence, the disulphide bridges in hGH provide stability, not biological function.

Clinical applications and further scientific questions

Mutations in GH1 affecting the cysteines in hGH

In our analysis of mutant hGH variants which cause GHD in affected patients, we found different underlying causes for the GHD.

hGH-R77C and hGH-S108C had only mildly reduced affinity to the GHR and only insignificantly reduced bioactivity in proliferation assays compared to hGH-wt. Both variates however, where expressed and secreted at a significantly lower rate in pituitary cells compared to hGH-wt. It is reasonable to speculate, that the misfolding of the hGH molecule due to the aberrant disulphide bridges is detected by the pituitary cell`s secretory machinery and that therefore, the pulsatile secretion of the mutant hGH upon stimulation is impaired; while the hGH molecule itself is functional.

C182X-hGH displayed significantly reduced bioactivity and stability compared to hGH-wt. This matches the clinical phenotype of the affected individual with severe GHD (25).

hGH-F176C showed only slightly reduced expression and secretion in pituitary cells with bioactivity and stability being very close to hGH-wt. It is therefore unsurprising, that the affected individual does not suffer from GHD.

hGH-C53S was observed to form disulphide-linked dimers in pituitary cells. To understand this this newly observed phenomenon in detail, we performed a number of follow-up experiments. Following is a summary of our findings, for further details please refer to our publication “Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation, Journal of Biological Chemistry”. We reproduced the formation of hGH-dimers for hGH-C53S in GC cells (rat somatotropic cells) and observed, that additional omission of p.C165 to hGH-C53S-C165A blocks the formation of hGH-dimers, providing evidence that the hGH-dimers are linked via p.C165. We then attempted to generate sufficient amounts of this hGH-dimer to study its characteristics, however as the dimer is not formed in other mammalian cell types we studied and only expressed in low quantities in pituitary cells, this was initially not successful. We therefore generated vectors to express hGH-C53S in E. coli cells, and were able to purify high amounts of hGH-C53S-dimers and hGH-wt from E. coli cells genetically modified to enable disulphide formation in the cytoplasm. Using Ni2+-affinity chromatography and his-tagged hGH followed by immunoaffinity chromatography and size-exclusion chromatography we were able to isolate hGH-C53S dimers. This dimer was found to be incapable of binding to the GHR and consequently was bioinactive in cell proliferation assays. Furthermore, in immunofluorescent imaging, hGH-C53S transfected pituitary cells did not form secretory granules as hGH-wt did and was instead restricted to the golgi apparatus of pituitary cells. Using a stimulus, release of GH-wt could be trigger in transfected AtT-20 cells, but not in GH-C53S transfected cells. Growth hormone deficiency related to bioinactive GH (Kowarski Syndrome) caused by the formation of a hGH-dimer is a newly described phenomenon.

Overall, this extensive study on the underlying mechanisms how mutations in GH1 affecting the cysteines in hGH can cause growth hormone deficiency reveal a variety of mechanisms.

The disulphide bridges in rhGH

rhGH is the preferred treatment for GHD and also in cases of idiopathic short statue, since it was first synthesised and made commercially available in 1985 (31). Today, rhGH manufactured for medicinal

purpose is expressed in E. coli cells and subsequently purified. The exact procedure is still subject of numerous recent studies, evaluating different expression systems and procedures. The process of harvesting hGH from inclusion bodies often relies on procedures involving reducing chemicals, which destroy disulphide bonds (32). As shown in our study, hGH devoid of disulphide bridges has moderately, but significantly reduced bioactivity compared to hGH-wt. The resilience of the hormone to proteolytic degradation however was strongly affected, which reduces the biological half-live of the hormone. As such, the disulphide bridges in hGH are essential for its biological function and our study provides further evidence that careful attention to the integrity of the disulphide bridges should be paid when generating recombinant hGH for treatment of GHD. Furthermore, we developed a new, efficient method to express hGH with intact disulfide bonds in the cytoplasm of genetically modified E. Coli cells including a procedure for purification. This enabled the generation of mg of hGH in a time and cost-effective manner. For details on yields and protocols we refer to our study published as "Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation" in the Journal of Biological Chemistry.

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Eidesstattliche Versicherung

„Ich, Max Sander, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: The molecular mechanisms underlying Inherited Growth Hormone Deficiency caused by cysteine mutation, Die molekularen Mechanismen des angeborenen Wachstumshormonmangels verursacht durch Cystein-Mutationen, selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Max Sander hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Max Sander, Zida Wu, and Christian J. Strasburger, Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation, Journal of Biological Chemistry, 2020.

Durchführung aller dargestellten Experimente einschließlich der Handhabung der Zellkulturen, mit Ausnahme eines Teils der Western-Blots von Figure 4.
Etablierung der Verfahren in unserem Labor und Entwicklung der Protokolle für die Immunhistologie, die Proteinexpression in E. Coli, die hGH-Aufreinigung aus E. Coli, die Generierung der Vektoren für die his-tagged Varianten, die De-Glykolisierungsexperimente und die Wachstumshormonsekretionstimulationsexperimente mit AtT-20 Zellen in unserem Labor. Weiterentwicklung und Optimierung vorhandener Protokolle für die anderen durchgeführten Experimente.
Durchführung aller statistischen Analysen und Herstellung aller enthaltenen Abbildungen und Tabellen.
Schreiben des Manuskriptes und Korrespondenz mit den Reviewern und dem Journal.

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Unterschrift, Datum und Stempel des erstbetreuenden Hochschullehrers

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Unterschrift des Doktoranden/der Doktorandin

Auszug aus der Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI Selected Categories: **"BIOCHEMISTRY and MOLECULAR BIOLOGY"** Selected Category Scheme: WoS

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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	75,461	32.621	0.171980
2	CELL	230,625	31.398	0.583260
3	Annual Review of Biochemistry	19,873	20.154	0.030170
4	TRENDS IN BIOCHEMICAL SCIENCES	16,944	15.678	0.030830
5	MOLECULAR CELL	61,604	14.248	0.181170
6	Nature Chemical Biology	19,562	13.843	0.061240
7	NATURE STRUCTURAL & MOLECULAR BIOLOGY	27,547	13.333	0.081820
8	TRENDS IN MICROBIOLOGY	11,344	11.776	0.020970
9	MOLECULAR PSYCHIATRY	18,460	11.640	0.047200
10	NUCLEIC ACIDS RESEARCH	168,962	11.561	0.402470
11	NATURAL PRODUCT REPORTS	9,973	11.406	0.016090
12	TRENDS IN MOLECULAR MEDICINE	9,213	11.021	0.019720
13	EMBO JOURNAL	67,036	10.557	0.079780
14	MOLECULAR BIOLOGY AND EVOLUTION	44,664	10.217	0.101560
15	GENOME RESEARCH	38,842	10.101	0.105060
16	Molecular Plant	7,010	9.326	0.021690
17	CURRENT BIOLOGY	56,595	9.251	0.137200
18	PLOS BIOLOGY	28,750	9.163	0.058680
19	Cell Systems	1,129	8.982	0.009600
20	EMBO REPORTS	13,293	8.749	0.031350
21	Molecular Systems Biology	8,447	8.500	0.019830
22	PROGRESS IN LIPID RESEARCH	5,302	8.435	0.006750
23	PLANT CELL	48,393	8.228	0.063640
24	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	5,276	8.220	0.009300
25	MATRIX BIOLOGY	4,803	8.136	0.008500
26	CELL DEATH AND DIFFERENTIATION	18,865	8.000	0.031540
27	Molecular Cancer	10,301	7.776	0.017280
28	CURRENT OPINION IN CHEMICAL BIOLOGY	10,226	7.572	0.019010
29	MOLECULAR ASPECTS OF MEDICINE	5,157	7.344	0.009700
30	CURRENT OPINION IN STRUCTURAL BIOLOGY	10,619	7.179	0.024320
31	Redox Biology	4,083	7.126	0.012060

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
32	Molecular Ecology Resources	9,272	7.059	0.017390
33	ONCOGENE	66,411	6.854	0.075960
34	CELLULAR AND MOLECULAR LIFE SCIENCES	23,341	6.721	0.041340
35	ANTIOXIDANTS & REDOX SIGNALING	19,324	6.530	0.032120
36	CYTOKINE & GROWTH FACTOR REVIEWS	5,668	6.395	0.008050
37	Science Signaling	10,316	6.378	0.037220
38	MOLECULAR ECOLOGY	37,813	6.131	0.062120
39	FREE RADICAL BIOLOGY AND MEDICINE	40,089	6.020	0.043060
40	CHEMISTRY & BIOLOGY	12,036	5.915	0.022840
41	BIOMACROMOLECULES	36,807	5.738	0.040810
42	FASEB JOURNAL	41,572	5.595	0.051640
43	Cell Chemical Biology	936	5.592	0.005100
44	EXPERIMENTAL AND MOLECULAR MEDICINE	3,538	5.584	0.007100
45	ADDICTION BIOLOGY	3,947	5.578	0.009640
46	CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY	3,337	5.279	0.007160
47	RNA Biology	5,089	5.216	0.019400
48	Biochimica et Biophysica Acta-Gene Regulatory Mechanisms	7,047	5.179	0.019420
49	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR BASIS OF DISEASE	12,799	5.108	0.027740
50	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR AND CELL BIOLOGY OF LIPIDS	8,827	4.966	0.019590
51	Epigenetics	5,167	4.918	0.014110
52	STRUCTURE	14,417	4.907	0.036760
53	HUMAN MOLECULAR GENETICS	40,141	4.902	0.081220
54	JOURNAL OF MOLECULAR BIOLOGY	59,295	4.894	0.047560
55	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR CELL RESEARCH	15,355	4.651	0.035090
56	JOURNAL OF NEUROCHEMISTRY	37,022	4.609	0.030710
57	ACS Chemical Biology	10,139	4.592	0.038480
58	FEBS Journal	17,495	4.530	0.034500

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
59	JOURNAL OF LIPID RESEARCH	22,697	4.505	0.029380
60	RNA	13,110	4.490	0.029440
61	BIOCONJUGATE CHEMISTRY	15,194	4.485	0.021530
62	BIOESSAYS	9,697	4.419	0.016870
63	JOURNAL OF NUTRITIONAL BIOCHEMISTRY	9,815	4.414	0.014150
64	MOLECULAR PHYLOGENETICS AND EVOLUTION	18,604	4.412	0.029490
65	NITRIC OXIDE-BIOLOGY AND CHEMISTRY	3,680	4.367	0.004970
66	BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS	11,931	4.280	0.022190
67	ACS Chemical Neuroscience	4,336	4.211	0.013270
68	Computational and Structural Biotechnology Journal	933	4.148	0.003730
69	JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY	10,325	4.095	0.012650
70	Metallomics	4,240	4.069	0.009600
71	Journal of Genetics and Genomics	1,811	4.066	0.004100
72	International Journal of Biological Sciences	4,432	4.057	0.009970
73	AMYLOID-JOURNAL OF PROTEIN FOLDING DISORDERS	1,262	4.048	0.002420
74	CHROMOSOMA	3,527	4.021	0.005850
75	JOURNAL OF BIOLOGICAL CHEMISTRY	366,247	4.010	0.320840
76	METHODS	19,646	3.998	0.024790
77	APOPTOSIS	6,281	3.967	0.007390
78	BIOORGANIC CHEMISTRY	2,123	3.929	0.003030
79	INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES	20,621	3.909	0.030000
80	EXPERT REVIEWS IN MOLECULAR MEDICINE	1,790	3.865	0.001620
81	BIOCHEMICAL JOURNAL	47,538	3.857	0.037930
82	CURRENT OPINION IN LIPIDOLOGY	3,849	3.853	0.006100
83	MOLECULAR CARCINOGENESIS	5,244	3.851	0.007630
84	MOLECULAR MICROBIOLOGY	35,698	3.816	0.036170

Publikation

Sander, M., Wu, Z., & Strasburger, C. J. (2020). Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation. J Biol Chem, 295(15), 4893-4901. https://doi.org/10.1074/jbc.RA119.009101

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publikationsliste

"Short stature explained by dimerization of human growth hormone induced by a p.C53S point mutation", Max Sander, Zida Wu, and Christian J. Strasburger, J. Biol. Chem. jbc.RA119.009101. doi:10.1074/jbc.RA119.009101

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