Aus der Universitätsklinik für Kinder- und Jugendmedizin Tübingen
Abteilung Kinderheilkunde I mit Poliklinik
Ärztlicher Direktor: Professor Dr. D. Niethammer
Sektion Pädiatrische Endokrinologie
Leiter: Professor Dr. M.B. Ranke

Structural Analysis of Human Growth Hormone with Respect to the Dominant Expression of GH Mutations in Isolated Growth Hormone Deficiency Type II

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Daniel I Iliev

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Dekan:	Professor Dr. C. D. Claussen
1. Berichterstatter:	Privatdozent Dr. G. Binder
2. Berichterstatter:	Privatdozent Dr. R. Lammers

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Contents

1. Abbreviations:

aa – amino acid
AIDA – advanced image data analyser
ATCC – American type culture collection
cDNA – complementary DNA
CE – cell extract
Ct – threshold cycle
ER – Endoplasmic reticulum
ESPE – European Society for Paediatric Endocrinology
GH – growth hormone
GHD – growth hormone deficiency
hGH – human GH
IGFBP – insulin-like growth factor binding protein
IGHD – isolated growth hormone deficiency
M – medium
MPHD or CPHD – multiple or combined pituitary hormone deficiency
rhGH – recombinant hGH
RIA – radioimmunoassay
wt – wild type

2. Abstract

Human GH protein consists of four alpha-helices and contains two disulfide bridges. Isolated growth hormone deficiency type II (IGHD II) is mainly caused by heterozygous splice site mutations of *GH-1* leading to the disruption of one disulfide bridge (Cys53-Cys165) and to the loss of amino acids (aa) 32-71 which comprise the complete loop between alpha-helices 1 and 2. The mutant GH protein exerts a dominant-negative effect on wild-type (wt) GH secretion by unclear mechanisms.

For the study of the structure-function relationship of GH mutants concerning the dominant-negative effect, expression vectors harbouring mutated GH cDNAs (the mutations affecting either the linker region between alpha-helices 1 and 2 or the disulfide bridge Cys182-Cys189) were transiently cotransfected with a vector encoding wtGH (pwtGH) into GH_4C_1 cells. Plasmids encoding either β-galactosidase, luciferase or IGFBP-2 were cotransfected with pwtGH and either of the GH mutants.

For the study of a potential dominant-negative effect due to disturbed Zn^{2+}-binding, expression vectors harbouring mutated GH cDNAs were constructed in which triplets encoding histidine and glutamine residues were mutated to triplets encoding alanine residues. These plasmids were transiently cotransfected with a vector encoding wtGH (pwtGH) into GH_4C_1 cells.

Compared to the control transfection with pwtGH, GH secretion was mildly decreased by coexpressing wtGH and different GH point mutants with isolated disruption of the disulfide bridge Cys53-Cys165. Similar results were observed with GH mutants deleted in aa 32-46 or 32-52. Deletion of more aa (32-53, 32-63, 32-69, 32-71) ascendingly decreased GH secretion and content of GH in parallel with the increasing length of the deleted stretch. Disruption of the disulfide bridge constituted between Cys182-Cys189 in the fourth alpha-helix of GH protein (mutant del188-190GH) did not show to play a role in the exertion of the dominant-negative effect. Partial or complete disturbance of Zn^{2+}-binding by amino-acid substitutions in the responsible

domains also did not reveal to participate in the expression of the dominant-negative effect.

An inhibitory dose-dependent effect of del32-69GH and del32-71GH on the activity/amount of coexpressed β-galactosidase, luciferase, and IGFBP-2 was found while mRNA levels were unaffected.

Hence, the extent of deletion in the linker region between alpha-helices 1 and 2 played the major role in expression of the dominant-negative effect. The inhibitory effect of GH mutants on heterologously expressed non-GH proteins suggests that the dominant-negative effect is not limited to GH and not even to proteins of the regulated secretory pathway but may depend on expression levels.

3. Introduction

A pituitary-derived factor which stimulated growth was described for the first time in 1921 (1). It exerted a considerable effect upon growth, maturity, and oestrus cycles in rodents when applied intraperitoneally. Two decades later, in 1944 Li and Evans isolated for the first time anterior pituitary growth hormone (GH) (2). Since the early 1960s human growth hormone (hGH) has been used as a substitutive therapy in children with growth hormone deficiency (GHD) (3, 4). Shortly after that the scope of applications for the GH expanded and since the late 1960s it has been used also for the treatment of disorders unrelated to GHD in which the main sign and symptom is short stature (5).

GHD can be isolated (IGHD) or a part of multiple (or combined) pituitary hormone deficiency (MPHD or CPHD). In the former case the only endocrine disorder is the insufficient or completely lacking production of GH, while in the latter – GHD is combined with deficient production of other pituitary hormones.

In a large number of cases severe GHD is familial (6, 7) and has a genetic basis. Recent advances in the knowledge of pituitary development have enhanced the understanding of the etiology of IGHD and CPHD (8). Mutations of the key pituitary transcription factors: *POU1F1* (*Pit-1*) whose transcript binds to the *GH-1* promoter and also to other target genes, such as those encoding prolactin and TSH, is a newly discovered cause for MPHD (9). Mutations of *PROP1* (10, 11) and *LHX3* (*P-Lim*) (12), result in an aberrant development of the pituitary gland and thus, MPHD. Mutations in *HESX1* expressed during the development of Rathke's pouch, may also lead to MPHD, and in addition – to severe developmental abnormalities, such as septo-optic dysplasia (13).

IGHD occurs at an estimated incidence of 1 in 3500-10000 live births (14). Most of the cases are sporadic but an estimated 5-30% are familial (i.e. IGHD in two or more first degree relatives). Monogenetic GHD is subclassified in three groups: autosomal recessive (IGHD I), autosomal dominant (IGHD II) and X-chromosomal (IGHD III) (15). The group with IGHD I is further subdivided in patients with a total absence of GH (IGHD IA) and those with a severe GH deprivation (IGHD IB). Southern Blot analysis demonstrated that the children with IGHD IA have a homozygous *GH-1* gene deletion and, as a consequence, no genetic information for GH (16). The healthy parents of these children are hemizygous for *GH-1*, showing that the presence of a single *GH-1* allele is sufficient for GH production and secretion resulting in normal GH levels in serum (17). The genetic basis for the most common type of inheritance IGHD IB is unclear in most cases. In few patients splice site mutations in intron IV of *GH-1* were described, resulting in skipping of the nucleotides coding for amino acids (aa) 103 to 126 and subsequently in a frame shift in exon 5 (18). Inactivating mutations of the gene encoding the growth hormone releasing hormone receptor were found as an alternative genetic basis of IGHD IB (19).

Isolated growth hormone deficiency type II (IGHD II) has mainly been described in patients harbouring mutations in the intron III donor splice site of one *GH-1* allele, which cause skipping of exon 3 (20, 21, 22, 23). The resulting gene product lacks amino acids 32-71 and, therefore, the entire loop connecting the first alpha-helix of the GH molecule to the second one (24) (Fig. 1).

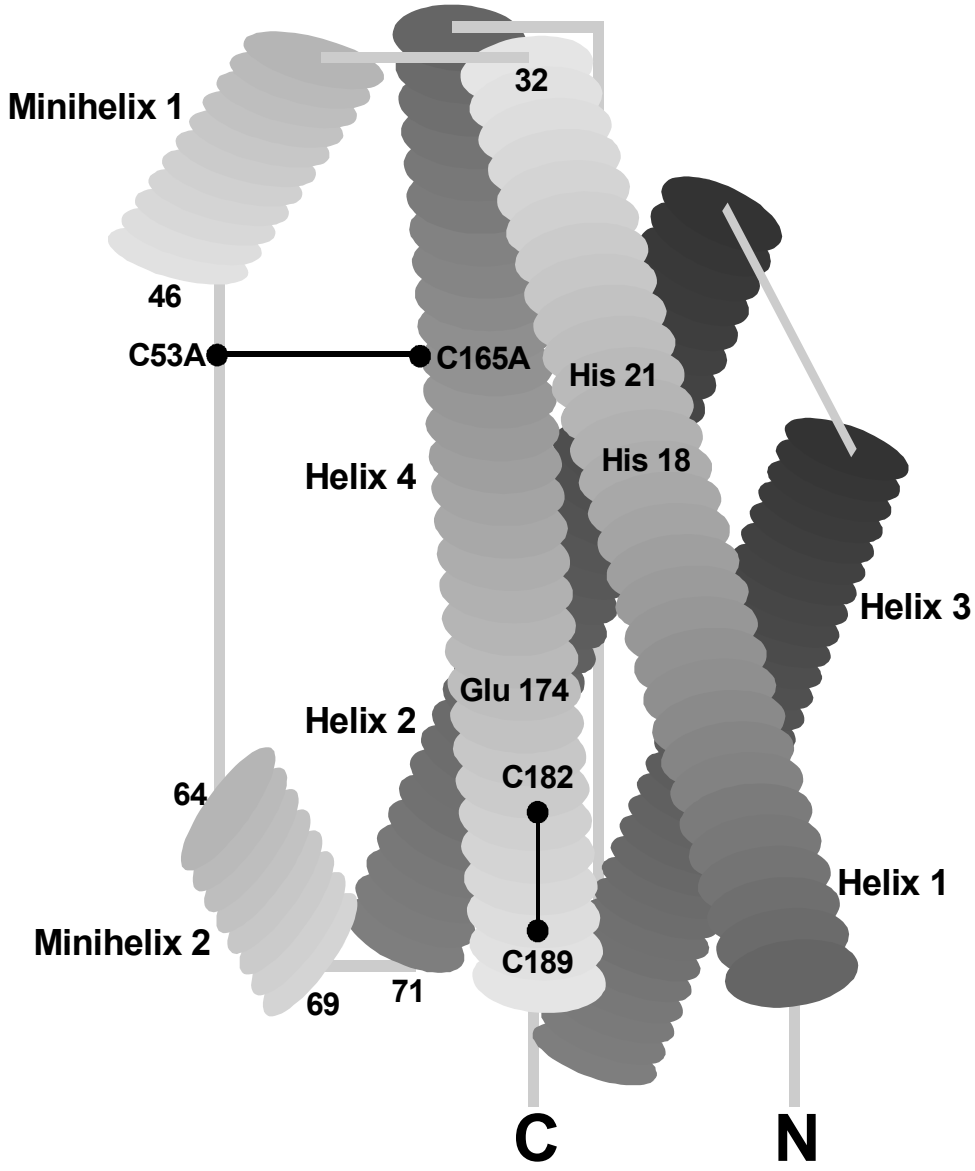

Figure 1

Schematic representation of the wtGH protein tertiary structure. The two intramolecular disulfide bridges and distinct aa positions corresponding to endpoints of deleted aa stretches are outlined.

The mutant GH protein is thus subject to serious structural changes. In patients harbouring this kind of mutation GH concentrations are extremely low even though one intact *GH-1* allele is present. Therefore, the presence of del32-71GH in the somatotrophs causes a blockade of wild type (wt) GH secretion by a still unknown mechanism (25, 26). In contrast, the splicing variant del32-46GH (the 20 kD GH variant), which also lacks a part of the loop connecting alpha-helices 1 and 2 (24) (but possibly without a major impact on the protein structure) accounts for approximately 10% of GH found in the serum of normal individuals and evidently does not have any adverse effects on the secretion of wtGH (27).

In addition to the intron III splice site mutations, three dominantly acting point mutations have been described in IGHD II patients which lead to exchanges of highly conserved amino acids and also suppress wt GH secretion to varying degrees (P89L, V110F, R183H; 28, 29, 30, 31). All three concerned amino acids are located at protruding sites of the tertiary structure of the hGH molecule and are possibly engaged in intramolecular interaction of the four alpha helices or in interaction with other GH molecules (24). On the contrary, other known missense mutations, frame shift mutations in intron IV (frame shift 56-131, frame shift 103-197) (21, 32) or hemizygosity for *GH-1* display a recessive phenotype (27, 33).

A recent study has demonstrated the plausible crucial role of the integrity of the disulfide bridge constituted between amino-acid residues Cys182-Cys189 on the alpha helix 4 of hGH molecule for the exhibition of the dominant-negative effect in IGHD II (34). Patients bearing a heterozygous deletion in exon 5 of *GH-1* (del188-190), thus encoding a protein missing the cysteine residue at position 189, presented with short stature, moderately low response of GH secretion to stimulation tests, as well as relatively low levels of IGF-I and IGFBP-3. It has been hypothesized that an unpaired cysteine residue of the mutated GH molecule could possibly affect GH secretion by interfering with folding and storage of both wt and mutant GH into secretory

vesicles. Finally, this disturbance might possibly cause death of the pituitary somatotrophs by affecting the secretory function of these cells.

Human prolactin, a protein related to human growth hormone (35), binds Zn^{2+} but the importance of this phenomenon is not completely elucidated and understood. It has been shown for prolactin that binding of Zn^{2+} is crucial for its intracellular processing and secretion (36). When GH_4C_1 neuro-endocrine cells were transfected with a plasmid encoding mutant human prolactin that does not bind Zn^{2+}, the secretion of the endogenously produced rat prolactin was greatly diminished and the amount of rat prolactin mRNA was decreased. In addition, the mutant human prolactin was not efficiently secreted and it was degraded intracellularly relatively quickly.

Recombinant human GH has also been shown to form soluble dimer zinc complexes. Histidine residues at positions 18 and 21 on alpha-helix 1 and glutamine residue at position 174 on alpha-helix 4 have been identified as zinc binding sites in the hGH molecule (37). Zn^{2+}-binding to hGH molecule serves at least two functions – first, it participates in the binding of hGH to its receptor linking the two proteins, and secondly, zinc binding stabilizes hGH structure (38). In the light of the findings above, it could be hypothesized that the dominant-negative effect exerted by some hGH mutants might also be influenced at least partly by zinc binding through decreasing protein stability and/or by disturbed aggregation of GH in secretory granules.

When human wtGH and del32-71GH were coexpressed in different cell types, the dominant-negative effect was observed only in neuroendocrine cells whereas no impairment of wtGH secretion was found in other cell types (e.g. COS, CHO, EBV-transformed lymphocytes; 39, 40, 41). Neuroendocrine cells differ from these cells by having specific modes of protein transport, sorting, storage and release (42, 43). In transient transfection studies involving rat GH_4C_1 neuroendocrine cells it was observed that del32-71GH suppresses both the intracellular accumulation and secretion of wtGH without itself being

accumulated or secreted, while wtGH and del32-71GH mRNAs were shown using Northern analysis to be present in approximately equal quantities (41). Studies with COS cells revealed an uneven distribution of wtGH and del32-71GH, the former being localised in the Golgi apparatus and the latter retained in the endoplasmic reticulum. It was suggested that the presence of the misfolded protein causes Golgi apparatus fragmentation, thus disrupting the transport from the endoplasmic reticulum to the Golgi apparatus involving also other membrane and secretory proteins, namely thyrotropin-releasing hormone receptor, rat prolactin and secreted alkaline phosphatase (44). These findings in non-neuroendocrine cells were not observed in GH_4C_1 neuroendocrine cells showing no deleterious effect of human del32-71GH on the production and secretion of heterologously expressed human prolactin. This fact suggests that effects of mutant GH are cell-type specific (41). In transgenic mice expressing human del32-71GH a marked decrease in GH levels was found in pituitary extracts and the affected animals developed short stature (45). Moreover, multiple anterior pituitary deficiencies, pituitary hypoplasia, morphological abnormalities of somatotrophs with few secretory vesicles, and macrophage hypophyseal invasion were detected. In contrast, the pituitary abnormalities observed in patients with IGHD II are not as severe as those evident in the *in-vivo* mouse model since low to normal size of the pituitary gland was confirmed by magnetic resonance tomography analysis (46). In a recent clinical study, however, secondary hypocortisolism and hypothyroidism were observed in some patients affected by IGHD II. Until recently, it had been believed that in humans the IGHD II was basically isolated; the lately observed in some patients latent secondary hypocortisolism and hypothyroidism makes the term “isolated” not quite appropriate for the whole group (manuscript in preparation).

Several hypotheses have been discussed in the literature towards explaining the basic mechanisms of the interference of some mutant GH forms with wtGH. These include (i) accumulation of toxic aggregates of mutant proteins; (ii) decrease of intracellular stability of wtGH due to cellular responses

induced by unfolded proteins (overload response, unfolded protein response, induction of cell type specific degradation systems); (iii) a specific blockade of GH aggregation and/or sorting into secretory granules; and (iv) impaired maturation of secretory granules (47).

4. Aim of the study

The aim of this *in-vitro* study using GH_4C_1 cells was to elucidate the importance of specific amino acids or stretches of amino acids in the context of the GH tertiary structure for the exhibition of the dominant-negative effect.

Of particular interest was the loop connecting the first and the second α-helices in the hGH molecule. The majority of the patients with isolated growth hormone deficiency type II bear a heterozygous mutation encoding a protein product in which this loop is missing.

Of additional interest for the study was to elucidate the importance of amino-acid residues situated at protruding sites of the tertiary structure of the hGH molecule and hence possibly engaged in preserving the right conformation, respectively taking part in the intramolecular interaction of the four alpha helices or in interaction with other GH molecules. These were regions presumably crucial for aggregation or dimerization, e.g. histidine and glutamine residues involved in Zn^{2+}-binding (His18, His21, Glu174) and the cysteine residues necessary for the formation of the second disulfide bridge in helix 4 (Cys182-Cys189).

5. Materials and methods

5.1. DNA Vectors

Deletion and point mutations of GH cDNA were performed using overlap extension PCR technology (48). WtGH cDNA (49) inserted in a pcDNA3-vector (pwtGH) (kindly gifted by P. Dannies) was used as a template. The oligonucleotides used for site-directed mutation were:

C53Afor: 5'-GAC CTC CCT CGC ATT CTC AG-3';

C53Arev: 5'-CTG AGA ATG CGA GGG AGG TC-3' (for C53A-GH and C53A-C165A-GH);

C165Afor: 5'-GCT CTA CGC ATT CAG GAA GGA-3';

C165Arev: 5'-TCC TTC CTG AAT GCG TAG AGC-3' (for C165A-GH and C53A-C165A-GH);

del32-46for: 5'-CAG GAG TTT AAC CCC CAG ACC TCC CTC-3';

del32-46rev: 5'-CTG GGG GTT AAA CTC CTG GTA GGT GTC-3' (for del32-46-GH);

del32-52for: 5'-CAG GAG TTT TGT TTC TCA GAG T-3';

del32-52rev: 5'-ACT CTG AGA AAC AAA ACT CCT G-3' (for del32-52-GH);

del32-53for: 5'-CTA CCA GGA GTT TTT CTC AGA G-3';

del32-53rev: 5'-GAC TCT GAG AAA AAC TCC TGG T-3' (for del32-53-GH);

del32-63for: 5'-TAC CAG GAG TTT AGG GAG GAA-3';

del32-63rev: 5'-TTC CTC CCT AAA CTC CTG GTA-3' (for del32-63-GH);

del32-69for: 5'-CCA GGA GTT TAA ATC CAA CCT-3';

del32-69rev: 5'-GGT TGG ATT TAA ACT CCT GGT-3' (for del32-69-GH);

del188-190for: 5'-TCT GTG GAG GGC TTC TAG-3';

del188-190rev: 5'-CAG CTA GAA GCC CTC CAC AGA-3' (for del188-190-GH);

H18Afor: 5'-CGC GCC GCC CGT CTG CAC-3';

H18Arev: 5'-CAG ACG GGC GGC GCG GAG-3' (for H18A-GH);

H21Afor: 5'-CCA TCG TCT GGC ACA GCT G-3';

H21Arev: 5'-CCA GCT GTG CCA GAC GAT G-3' (for H21A-GH);

G174Afor: 5'-CAA GGT CGC CAC ATT CCT-3';

G174Arev: 5’-GGA ATG TGG CGA CCT TGT-3’ (for G174A-GH);

GH-5’-HindIII: 5’-GTT AAG CTT CCT GTG GAC AGC TCA C-3’;
GH-3’-XhoI: 5’-AGA CTC GAG TAT TAG GAC AAG GCT GGT-3’ (the last two were homologous to the 5’ and 3’ non-coding regions of the hGH cDNA including in addition *HindIII* and *XhoI* restriction sites, respectively).

In a first round of PCR amplicons were generated using either GH-5’-HindIII and one of the mutant reverse primers or GH-3’-XhoI primer and one of the mutant forward primers and wt GH cDNA as template. The PCR products were run on a 1% agarose gel, DNA bands were excised, and DNA was extracted using “QIAquick® Gel Extraction Kit” (Qiagen GmbH, Hilden, Germany). In a second PCR round the primers GH-5’-HindIII and GH-3’-XhoI were used with both purified amplicons of the first PCR round as template. The purified PCR products were cut with restriction enzymes specific for *HindIII* and *XhoI* sites (New England Biolabs GmbH, Frankfurt am Main, Germany), and subsequently purified after restriction digestion using “QIAquick® PCR Purification Kit” (Qiagen). They were cloned using T4 DNA Ligase (New England Biolabs GmbH) into the transfection vector pcDNA3.1 which contains the gene for Ampicillin resistance (Invitrogen GmbH, Karlsruhe, Germany). Plasmids were transformed into XL-1 blue supercompetent *E. coli* cells (Stratagene, Cedar Creek, USA) according to the manufacturer’s instructions. After transformation, the bacteria were seeded on agar plates with Ampicillin (100 µg/mL) and incubated at 37° C. 24 hours later a starter culture medium (LB medium supplemented with Ampicillin 150 µg/mL) was inoculated with a bacterial colony and plasmid DNA was isolated using “QIAprep Spin Miniprep Kit” (Qiagen). Screening for efficient cloning was done by use of restriction enzyme analysis (*HindIII* and *XhoI*). Ultimately, plasmid DNA was isolated from a suitable colony using “EndoFree Plasmid Maxi Kit” (Qiagen). The presence of the point or deletion mutation and the integrity of the hGH cDNA was verified by sequencing (GENterpise GmbH, Mainz, Germany).

5.2. Cell culture

GH_4C_1 cells used for the transfection experiments were purchased from the American Type Culture Collection (ATCC, LGC Promochem, Wesel, Germany). GH_4C_1 cells were cultured in D-MEM/F-12 (Gibco, Raisley, Scotland, UK) supplemented with 15% horse serum (Gibco) and incubated at 37°C and 5% CO_2 at air humidity of 100%.

5.3. Transfection experiments

The cDNA expression vectors generated, harbouring various mutant GH cDNAs, were cotransfected with pwtGH using the "Effectene® Transfection Reagent Kit" (Qiagen). Cotransfection experiments with expression vectors for β-galactosidase (pcDNA3.1.V5/His-lacZ, Invitrogen), firefly luciferase (pGL2LUC, Promega GmbH, Mannheim, Germany), or human insulin-like growth factor binding protein 2 (pcDNA3.1-IGFBP-2) were performed. For transfection, 5 x 10^5 cells were seeded in 60 mm poly-D-lysine coated transfection dishes (BD Biosciences, Meylan Cedex, France). Transfection was carried out according to the manufacturer's instructions 24 hours after seeding at approximately 80% confluency. A total amount of 1 µg DNA and a DNA ratio of wtGH to mutant GH of 1:1 was used, if not otherwise stated. If the amounts of mutant constructs were varied, the total amount of the transfected plasmid DNA was adjusted to 1 µg DNA using an empty pcDNA3.1 vector. 48 hours post-transfection medium and cells were harvested and cellular proteins were extracted using Reporter Lysis Buffer (400 µL/culture dish) according to the manufacturer's instructions (Promega, Madison, USA). Shorter or longer incubation periods (18, 24, and 72 hrs, respectively) were demonstrated to be inappropriate for the radioimmunoassay (RIA) used as the measured GH values were beyond the detection capacity of the system being either lower than 1 ng/mL or higher than 300 ng/mL.

5.4. RNA extraction and Real-time RT-PCR

Total RNA was isolated 24 hours post-transfection using the RNeasy Mini Kit (Qiagen) with a simultaneous on-column DNase digestion with the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. Additionally RNA solution was treated with DNaseI RNase free (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR (Promega) performed with or without reverse transcriptase showed presence of hGH transcripts and efficient removal of plasmid DNA. cDNA was synthesized using Omniscript RT kit (Qiagen). Consecutively Real-time RT-PCR was performed in a Bio-Rad iCycler using the SYBR Green Supermix (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The primers were designed to yield products no longer than 150 bp. For each sample, duplicate measurements were performed and the arithmetic mean was calculated from each duplicate measurement. Ct (threshold cycle) values were obtained using the Bio-Rad iCycler Software version 3.0. Results were finally analysed using the Microsoft Excel Software.

5.5. hGH and IGFBP-2 measurements

GH values were measured in medium and cell lysate using a radioimmunoassay (RIA) specific for hGH (NIBSC Code: 88/624, polyclonal antibodies applied, sensitivity 0.05 ng/mL, intraassay variability 6.7% at 1.2 ng/mL, 7.9% at 2.4 ng/mL, 2.4% at 18.8 ng/mL) (50). IGFBP-2 values in media were measured using RIA specific for human IGFBP-2 as previously described (51).

5.6. Western blot analysis

15 µL media or cell extracts were separated on a 15% SDS polyacrylamide gel, blotted on an Immobilon –P Transfer Membrane (Millipore, Bedford, MA, USA) and subjected to antibody determination using either a rabbit polyclonal antiserum against recombinant hGH (Somatropin, NIBSC Code: 88/624) or a monoclonal antibody named 7B11 (kindly gifted by C. Strasburger, Berlin)

directed to the N-terminal domain of the hGH molecule. As secondary antibodies anti rabbit IgG (New England Biolabs GmbH) and anti Mouse IgG (New England Biolabs GmbH) were used. Chemiluminescence detection was performed using the ECL plus Western Blotting Detection System (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, England). Results were analysed densitometrically on a Raytest apparatus (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany) using the AIDA (advanced image data analyser) software, version 2.1. In addition the image was captured on Kodak film.

5.7. Cell counting

In experiments performed in parallel, 4, 24, 48, and 80 hours post-transfection the number of cells transfected either only with pwtGH or cotransfected with pwtGH and a del32-71GH expressing plasmid (pdel32-71GH) was estimated automatically (Cobas Micros, Roche, Montpellier, France, and Advia 120 Bayer, Holliston, MA, USA) as well as in a Neubauer-improved counting chamber (Brand, Wertheim, Germany). Vital and non-vital cells were distinguished under microscope via staining with Trypan blue solution, 0.4% (Sigma-Aldrich Co. Ltd., Irvine, UK).

5.8. β-galactosidase and luciferase activities

were determined in an automated luminometer Wallac 1420 Victor^2 ™ (Wallac Oy, Turku, Finland) using the β-Gal Reporter Gene Assay, chemiluminescent (Roche Diagnostics GmbH) and Luciferase Assay System (Promega, Madison, USA), respectively.

5.9. β-galactosidase staining

was performed directly on culture dish using reagents produced according to the instructions of the “In situ β-galactosidase staining kit” (Stratagene, La Jolla, CA, USA).

5.10. Statistical data analysis

Differences between the transfection groups were analysed by means of the Student's t-test.

6. Results

In order to investigate the structure-function relationship of hGH mutants with respect to a negative effect on wtGH secretion, a series of hGH constructs which were mutated or deleted in specific amino acids or stretches of amino acids was generated. We focused on the study of the linker region between alpha-helices 1 and 2 (Fig. 1), and in particular the stretch between aa 46 and 71 since the GH variant form del32-46GH (the 20 kD GH variant form) exerts no deleterious effect while del32-71GH has a strong adverse effect on wtGH secretion. Therefore, diverse deletion mutants, del32-46, del32-52, del32-53, del32-63, and del32-69 were constructed in which the respective amino-acid stretch was deleted. Of additional interest was the role of the disulfide bridge constituted between Cys53 and Cys165 which is disrupted in del32-71GH. Distinct point mutants were constructed in which the respective cysteine encoding DNA triplets were mutated to alanine encoding triplets, resulting in the GH mutants C53A, C165A and C53A-C165A (Fig. 1). The role of the disulfide bridge constituted between Cys182 and Cys189 was also of interest for the exertion of a potential dominant-negative effect. Therefore, the deletion mutant del188-190GH was constructed in which the respective amino-acid stretch was deleted (Fig. 1). The relevance of Zn^{2+}-binding of the hGH molecule for the production, processing, sorting into secretory granules, and secretion of GH was also studied. For this purpose a series of point mutants in which the triplets encoding the amino-acid histidine at positions 18 and 21, as well as triplets encoding the amino-acid glutamine at position 174 were mutated to triplets encoding the amino-acid alanine, thus preventing partially or completely Zn^{2+}-binding. The mutants were as follows: H18A, H21A, G174A, H18A-G174A, H21A-G174A, H18A-H21A, and H18A-H21A-G174A. In these mutants one, two or all three Zn^{2+}-binding amino-acids were substituted by the amino-acid alanine which does not bind Zn^{2+}.

GH_4C_1 cells were simultaneously transfected with pwtGH and one of the mutant GH expressing plasmids. 48 hours post-transfection GH amounts

were measured in incubation media and cell extracts. GH content in cells cotransfected with pwtGH/pwtGH, pwtGH/empty vector, pwtGH/pdel32-46, pwtGH/pdel32-52, pwtGH/pdel32-53, pwtGH/pdel32-63, pwtGH/pdel32-69, pwtGH/pdel32-71, pwtGH/pC53A, pwtGH/pC53A-C165A, pwtGH/pC165A, and pdel32-71GH/pdel32-71GH was determined using a hGH specific RIA (Fig. 2).

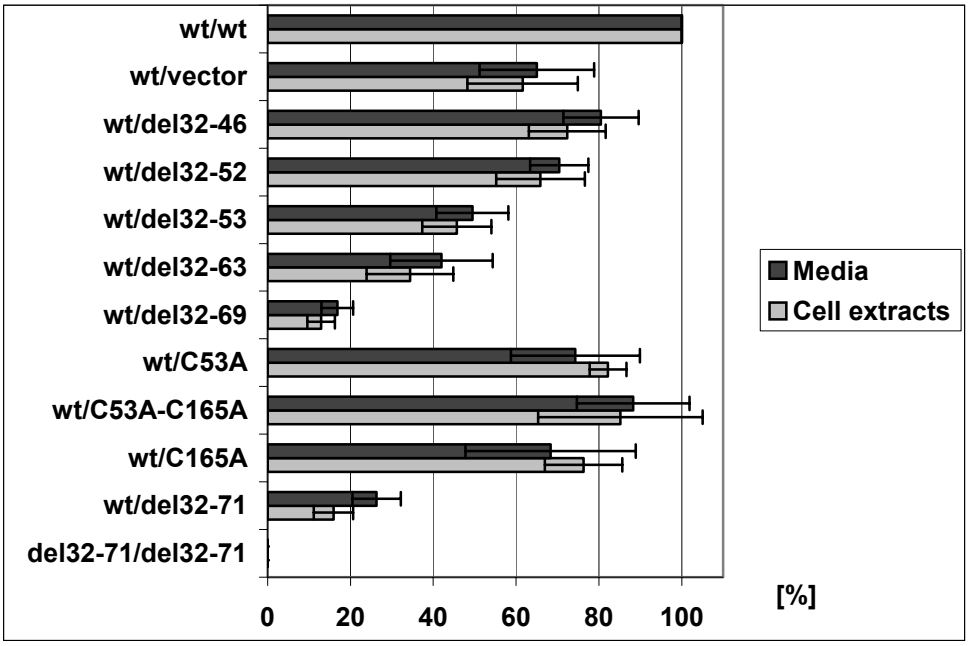

Figure 2

Relative GH concentrations in media and cell extracts of cotransfected GH_4C_1 cells measured using RIA. Mean values of 5 to 8 individual experiments +/-SD are represented as per cent of the values obtained for the pwtGH/pwtGH transfection which was set to 100%. The GH content of each sample was measured twice, using the mean value for calculations. P-values are given for the differences between pwtGH/pdel32-52GH and pwtGH/pdel32-53GH on the one side, and between pwtGH/pdel32-63GH and pwtGH/pdel32-69GH on the other side. The extent of deletion played the major role in the expression of the dominant-negative effect on wtGH.

In comparison to the control transfection (pwtGH/pwtGH set at 100%), the amount of hGH secreted into the medium was decreased by 35% when cotransfecting pwtGH/empty vector. It was decreased mildly by 15-30% when coexpressing wtGH and either of the point mutants C53A, C165A or C53A-C165A or mutants deleted in aa 32-46 or 32-52. Deletion of more aa (32-53, 32-63) led to a stronger decrease of secreted hGH by 50-60%. Interestingly, the difference in the GH output when coexpressing del32-52GH or del32-53GH with wtGH was statistically significant. This finding indicates that the disruption of the disulfide bridge Cys53-Cys165 in the context of the deleted stretch between aa 32 and 53 plays a role in the exertion of the dominant-negative effect. A severe decrease by 75-85% was observed when stretches 32-69 or 32-71 were deleted. Though not simply linearly correlated, the degree of GH reduction was proportional to the increase in size of the deletion. The same relationship between the extent of deletion and the amount of detectable hGH was found in protein extracts of the respectively transfected cells (Fig. 2). The amount of del32-71GH when singly expressed was below the detection limit of our RIA system.

In order to prove if the amount of hGH was underestimated in the RIA due to the intrinsic competition with recombinant hGH, cell extracts and media were analyzed using Western blots (Fig. 3).

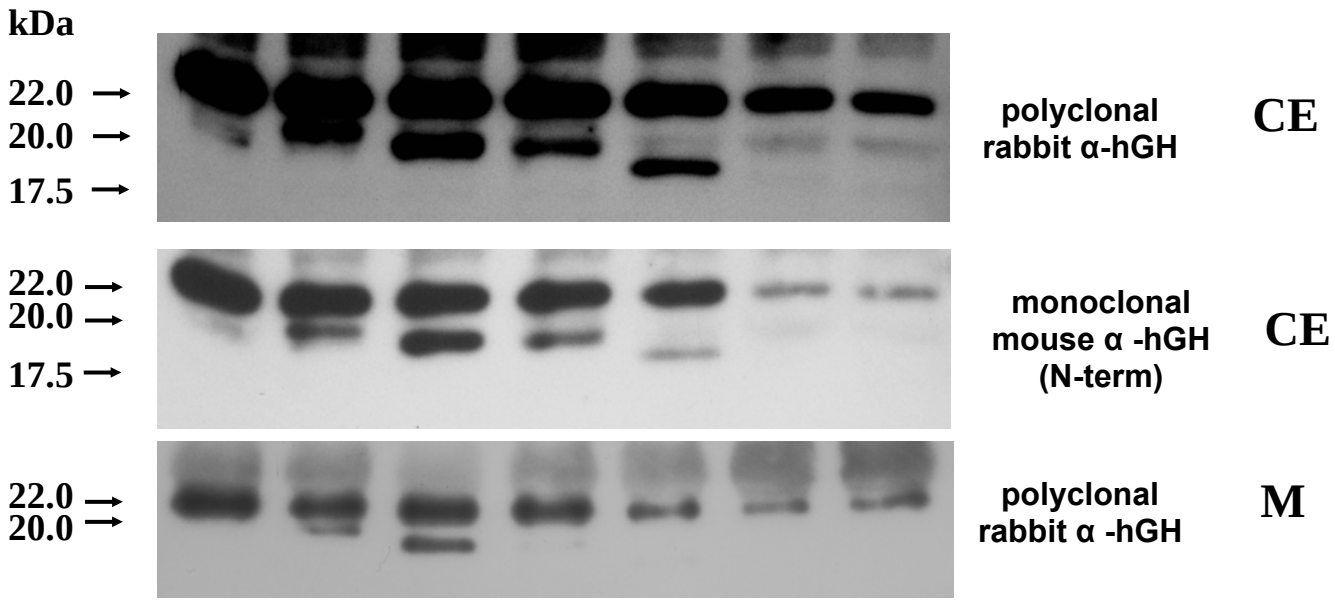

Figure 3

Western blot analysis of extracts (CE) or media (M) of cotransfected GH_4C_1 cells using a polyclonal rabbit anti-human GH antiserum (polyclonal rabbit α-hGH) or a monoclonal mouse anti-human GH antibody specific for the N-terminal domain (monoclonal mouse α-hGH). Protein bands corresponding to the wt and mutant hGH forms are depicted by arrows and the respective molecular weights are presented.

Densitometric evaluation of protein bands corresponding to wtGH and mutant GH (detected by polyclonal antiserum as well as by monoclonal antibodies directed towards the N-terminal domain of hGH) revealed comparable amounts as determined using RIA. The finding that mutant protein amounts were not underestimated in RIA was strengthened by the comparability of RIA and Western blot results for cells monotransfected with pwtGH, pdel32-46, pdel32-53, or pdel32-71 (Fig. 4), or pwtGH, pC53AGH, or pC165AGH (Fig. 5).

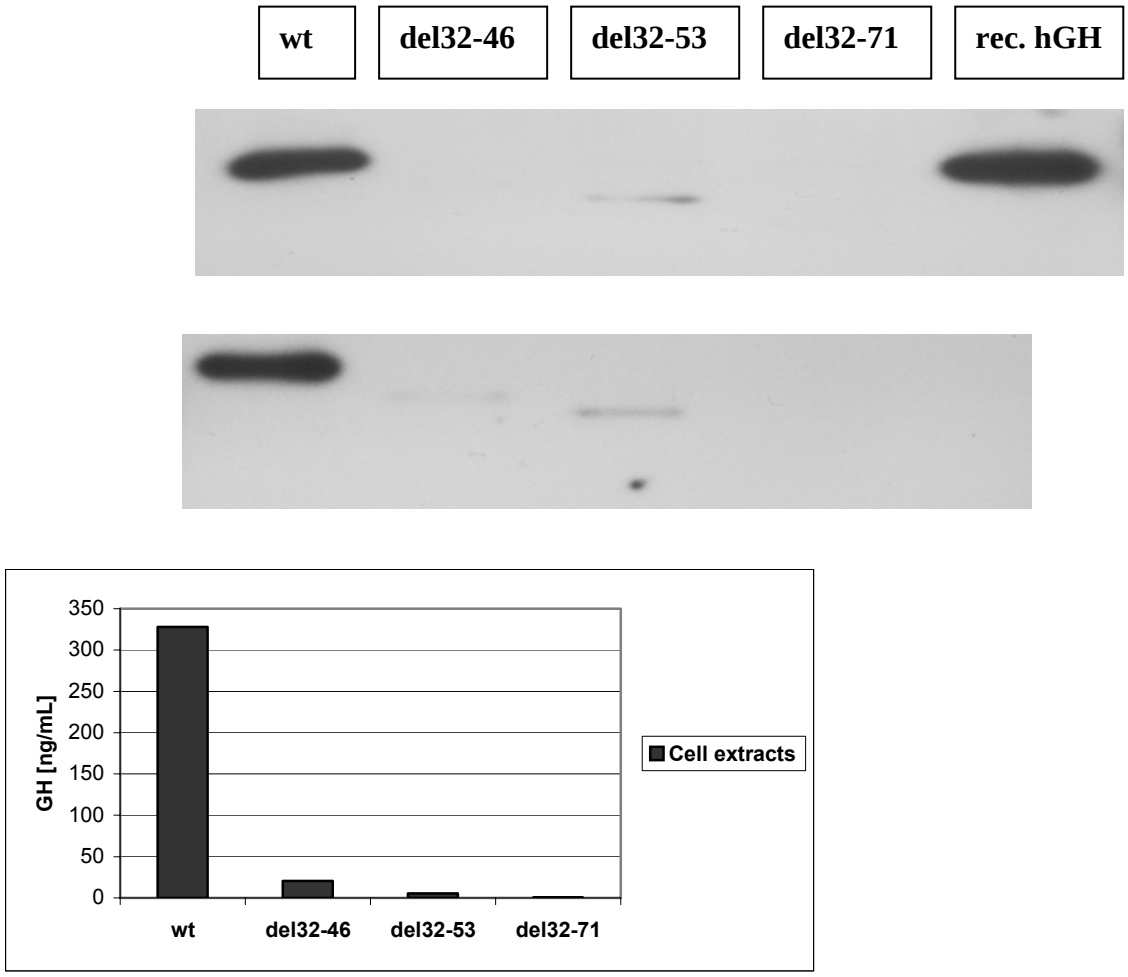

Figure 4

GH detected in lysates of GH_4C_1 cells monotransfected with a plasmid encoding either wtGH, del32-46GH, del32-53GH, or del32-71GH. The upper two panels represent data from Western blot analysis using a polyclonal rabbit anti-human GH antiserum (top) or a monoclonal mouse anti-human GH antibody specific for the N-terminal domain. The lower graph represents the GH content estimated by RIA using a polyclonal rabbit anti-human GH antiserum.

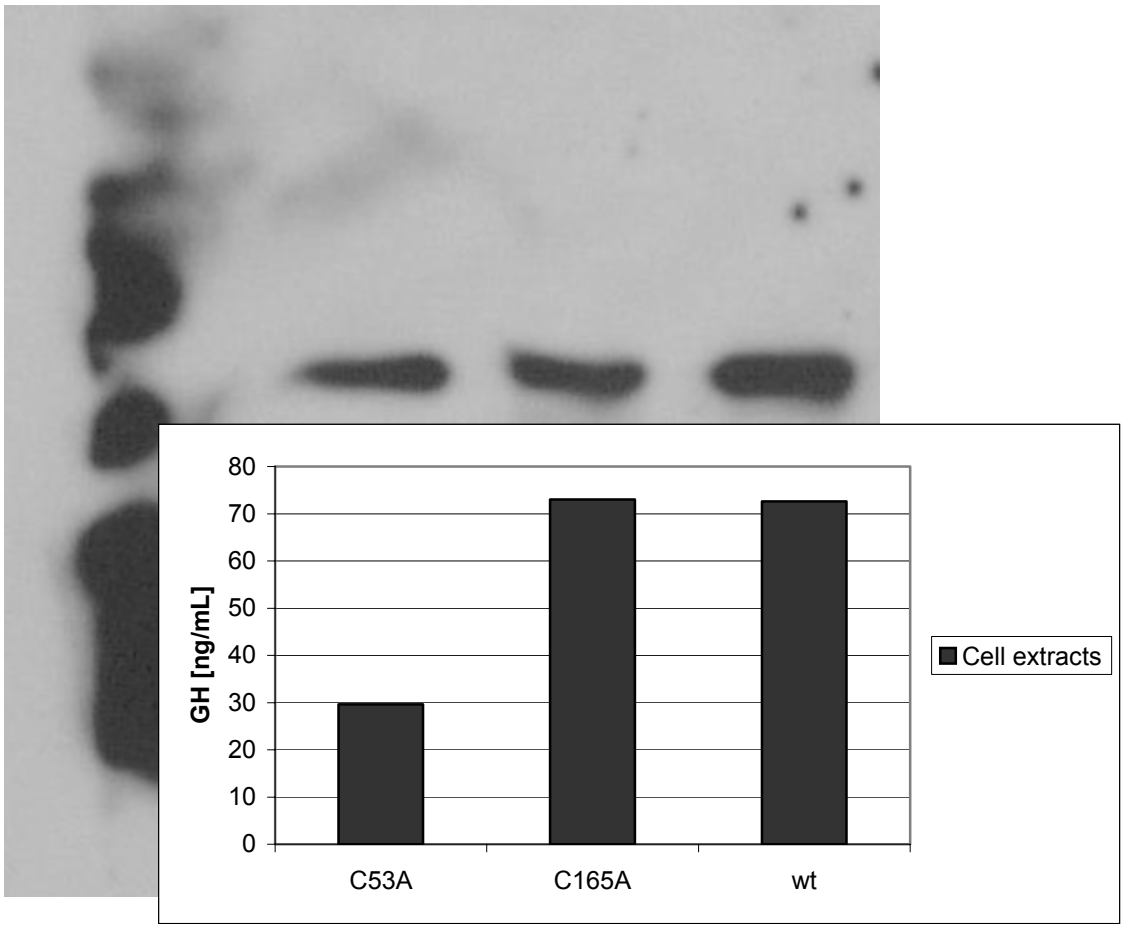

Figure 5

GH detected in lysates of GH_4C_1 cells monotransfected with a plasmid encoding either wtGH, C53AGH, or C165AGH. The upper panel represents data from Western blot analysis using a polyclonal rabbit anti-human GH antiserum. The lower graph represents the GH content estimated by RIA using a polyclonal rabbit anti-human GH antiserum.

Since the determined concentration of GH was slightly higher in experiments when pwtGH was cotransfected with an empty vector compared to cotransfection with pdel32-53GH in equivalent quantities (50%:50%) (Fig. 2), it was of interest to investigate for a dominant-negative effect beyond a mere dosage dependency exerted by del32-53GH. Therefore, in order to study if del32-53GH exerts a dominant-negative effect as suggested by the above RIA data, we performed parallel cotransfection experiments in which pdel32-53 or empty vector were cotransfected in varying amounts together with pwtGH (Fig. 6).

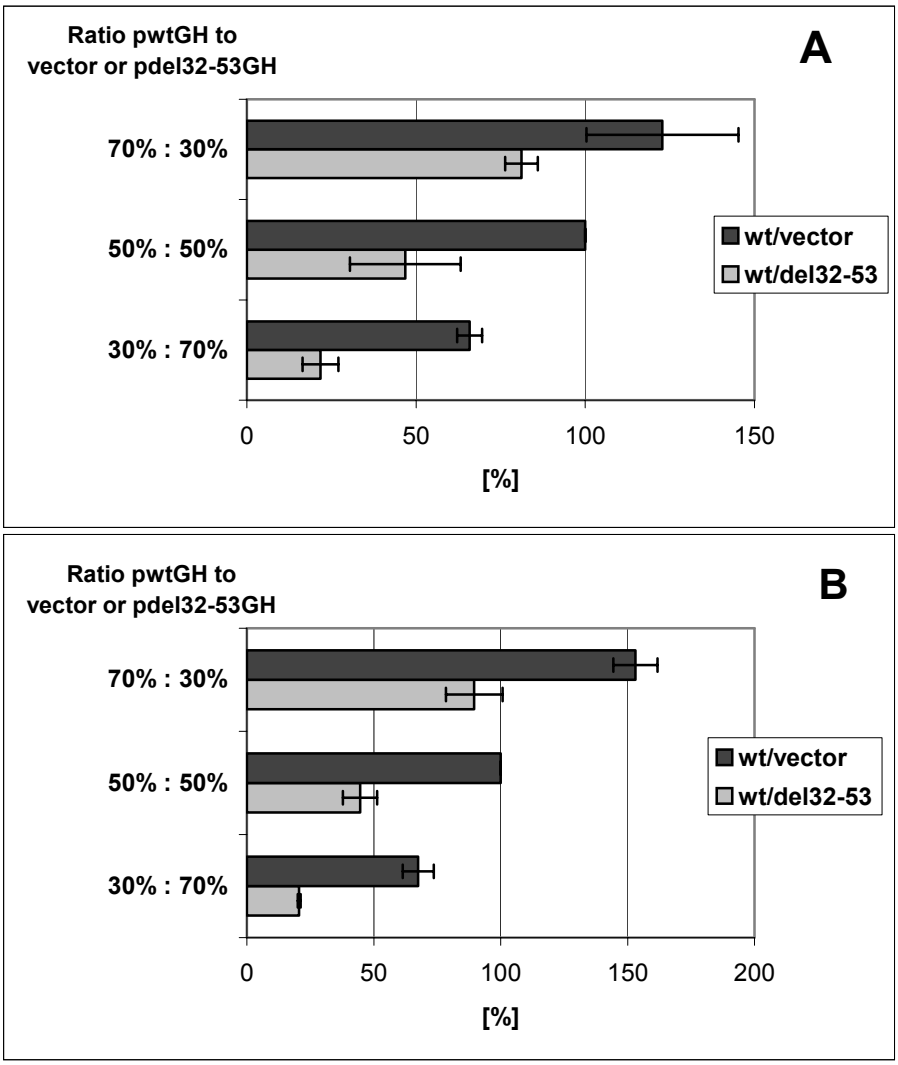

Figure 6

Relative GH concentrations in media (A) and cell extracts (B) of cotransfected GH_4C_1 (either with pwtGH/pdel32-53GH or with pwtGH/vector) measured using RIA. Mean values of 3 individual experiments +/-SD are represented as per cent of the values obtained for the pwtGH/vector cotransfection at equimolar amounts which was set to 100%. The GH content of each sample was measured twice, using the mean value for calculations. The ratio of the respective plasmids used for transfection experiments is depicted on the left hand side. The data reveal a dose-dependent dominant-negative effect of del32-53GH on wtGH.

The total amount of transfected plasmid was kept constant by adapting the amount of pwtGH cotransfected. The concentration of total GH was constantly lower for cells cotransfected with pdel32-53GH and pwtGH as compared to cells cotransfected with empty vector and pwtGH, thus indicating a dominant negative effect of del32-53GH on wtGH. This effect became significantly stronger with increasing amounts of pdel32-53GH cotransfected – cotransfection of increasing amounts of either pdel32-53GH or empty vector relative to pwtGH resulted in a much stronger decrease in GH concentrations detected for pwtGH/pdel32-53GH compared to pwtGH/empty vector from two thirds over less than a half to one third. This suggests an influence of the mutant protein on GH amounts beyond a mere dosage dependency.

The postulated important role of the second disulfide bridge constituted between Cys182 and Cys189 was not confirmed in our cell model system. Its disruption was not connected with any significant effect on the wtGH production and secretion (Fig. 7).

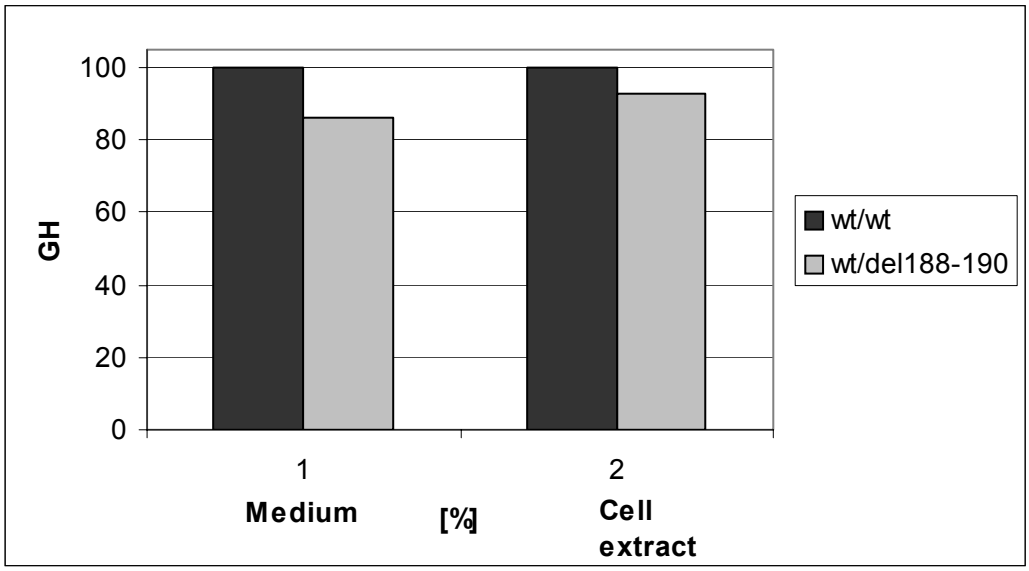

Fig. 7

Relative GH concentrations measured using RIA in medium and cell extract of GH_4C_1 cells either transfected with pwtGH or cotransfected with pwGH and pdel188-190GH in equimolar ratio. Values of a single experiment are represented as per cent of the values obtained for the pwtGH/pwtGH transfection which was set to 100%. The GH content of each sample was measured twice, using the mean value for calculations.

The role of Zn^{2+}-binding of hGH molecule was not proven to be crucial for the exertion of the dominant-negative effect. The working hypothesis of a slight effect exerted by the mono-mutants, a moderate effect exerted by the mutants with two aa exchanges and a strong dominant-negative effect exerted by the mutant with three aa exchanges (a mutant which does not bind Zn^{2+}) was not confirmed in our cell model system (Fig. 8).

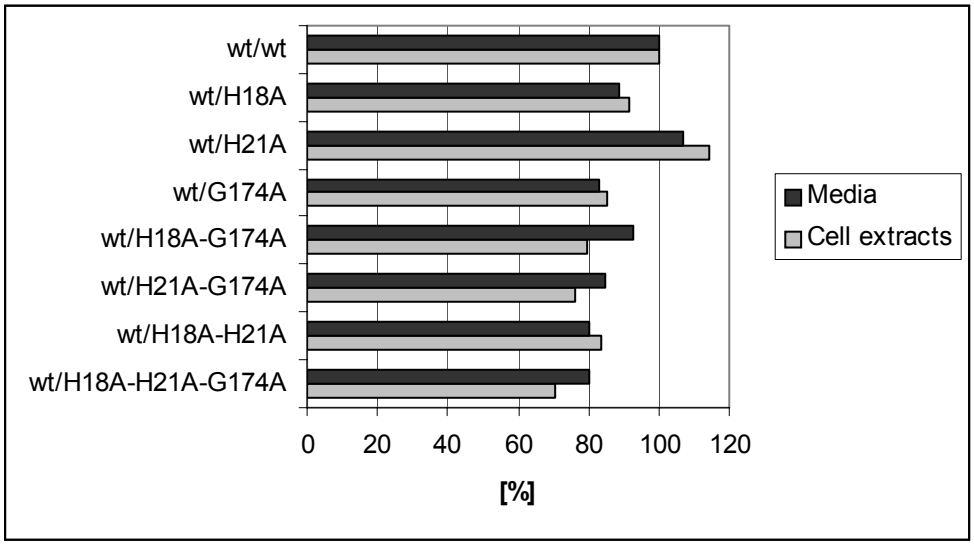

Fig. 8

Relative GH concentrations in media and cell extracts of cotransfected GH_4C_1 cells measured using RIA. Values of a single experiment are represented as per cent of the values obtained for the pwtGH/pwtGH transfection which was set to 100%. The GH content of each sample was measured twice, using the mean value for calculations.

The total cell numbers and the viability of cells expressing either only wtGH or coexpressing wtGH and del32-71GH stayed equivalent in individual experiments over periods of up to 80 hours, thus making an acute toxic effect of del32-71GH on the cells unlikely (data not shown).

The transfection efficiency was determined in individual experiments by counting the beta-galactosidase stained cells directly on culture dish and was found to be between 10 and 15%. No intraexperimental variation of transfection efficiency was found between cells transfected only with pwtGH or those cotransfected with pwtGH and either pdel32-46, pdel32-53, or pdel32-71GH. Interestingly, the intensity of the staining decreased with the increasing length of the deleted stretch (Fig. 9).

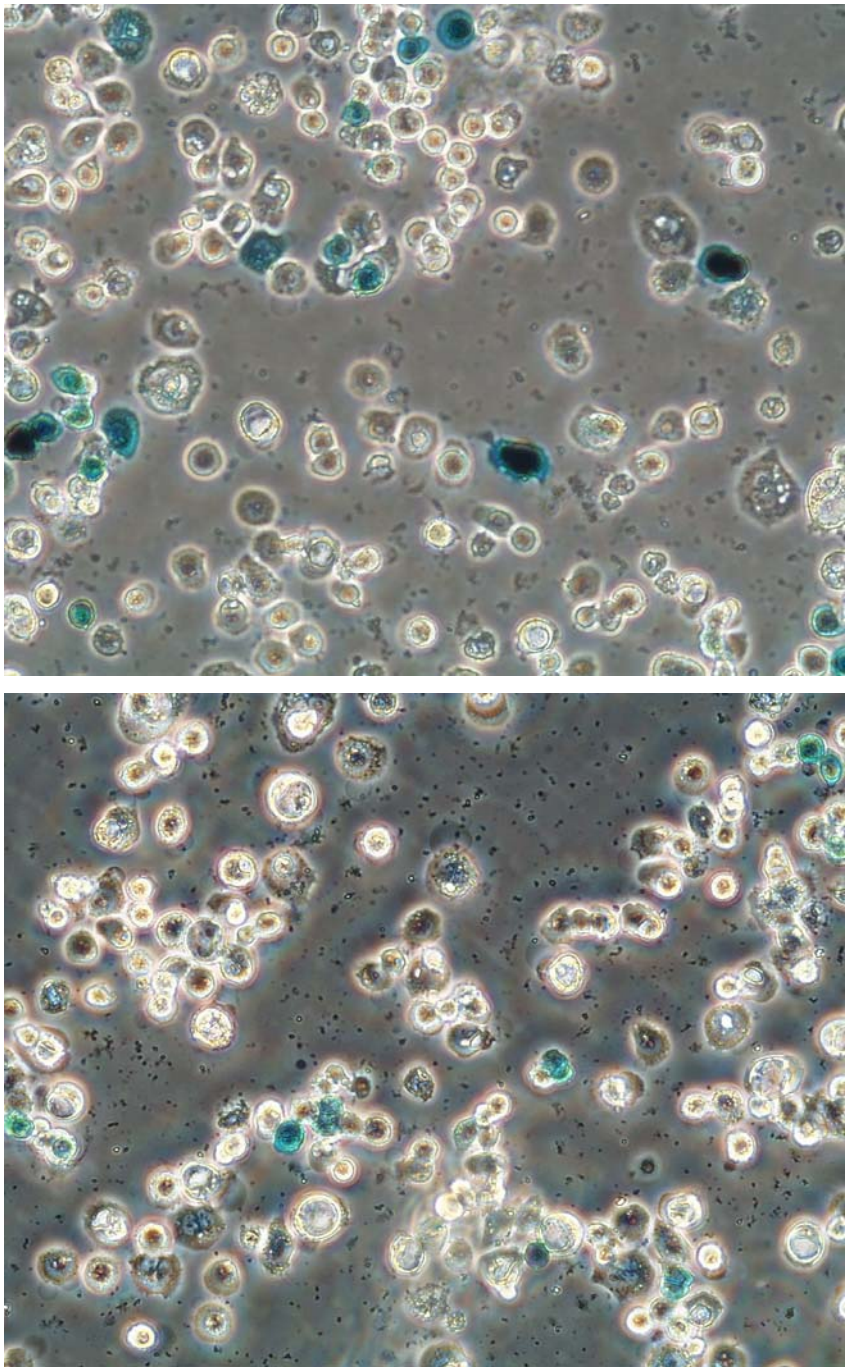

Fig. 9

Beta-galactosidase staining directly on the transfection dish performed 48 hours post-transfection. The upper picture represents cells transfected with pwtGH, the lower one – cotransfected with pwtGH and pdel32-71GH.

In order to normalize the values obtained by RIA measurements for transfection efficiency, plasmids containing cDNA encoding either β-galactosidase, firefly luciferase or IGFBP-2 were cotransfected and the activity or expression of the respective gene products was analyzed either in cell extracts (β-galactosidase and luciferase) or in media (IGFBP-2). Unexpectedly, the β-galactosidase activity measured in extracts from cells cotransfected with pwtGH/pdel32-71GH was approximately 5-fold lower in comparison to that of cells either transfected only with pwtGH or cotransfected with pwtGH and an empty vector (Fig. 10).

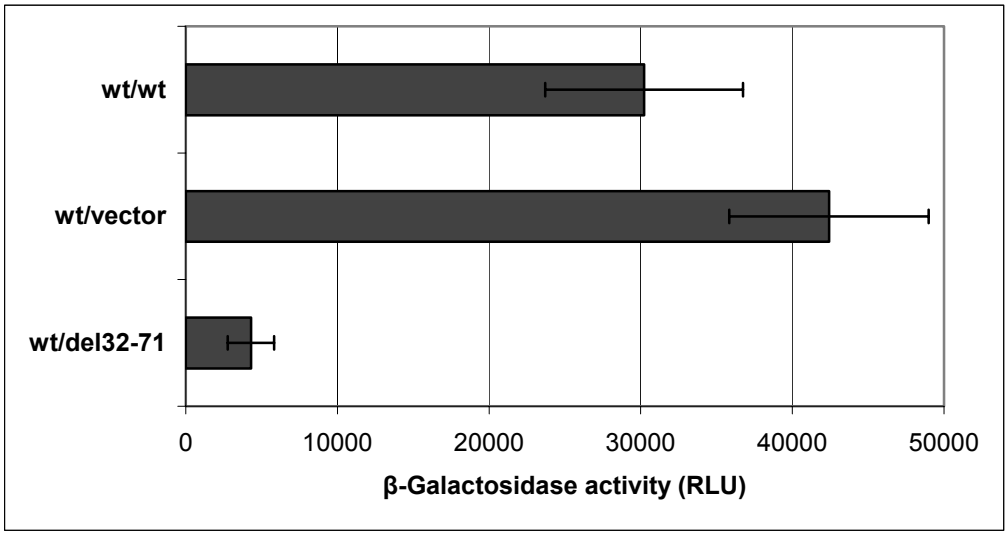

Figure 10

β-galactosidase activities in cell lysates. Cells were always cotransfected with 0.4 µg of each of the plasmids denoted and 0.2 µg of plasmid pcDNA3.1.V5/His-lacZ. Mean values +/-SD of triplicate transfections are presented. β-galactosidase activity was severely diminished in the presence of del32-71GH.

In addition, the dominant-negative effect of del32-71GH both on wtGH and on β-galactosidase appeared to be dose-dependent. When constant amounts of pwtGH and expression plasmid for β-galactosidase were cotransfected with increasing amounts of pdel32-71GH, a reverse correlation was found between the quantity of pdel32-71GH transfected on the one hand and the β-galactosidase activity (Fig. 11) and GH concentrations measured on the other hand (Fig. 12).

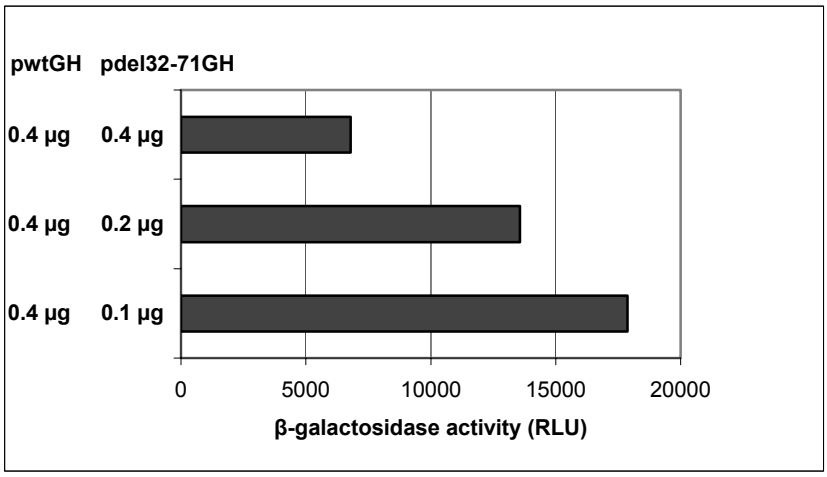

Figure 11

β-galactosidase activities in cell lysates. Cells were cotransfected with 0.2 µg pcDNA3.1.V5/His-lacZ, 0.4 µg pwtGH and 0.4, 0.2 or 0.1 µg pdel32-71GH. A total of 1 µg transfected plasmid DNA was achieved by cotransfection of respective amounts of an empty vector. Values represent the mean of duplicate determinations of one experiment.

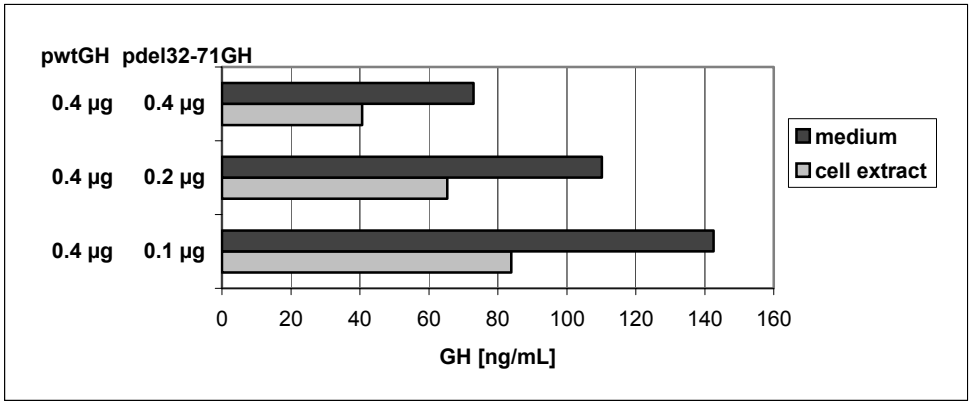

Figure 12

GH concentrations determined by RIA in media and cell extracts. Cells were cotransfected with 0.4 µg pwtGH, 0.4, 0.2 or 0.1 µg, pdel32-71GH and 0.2 µg pcDNA3.1.V5/His-lacZ. A total of 1 µg transfected plasmid DNA was achieved by cotransfection of respective amounts of an empty vector. Values represent the mean of duplicate determinations of one experiment.

Similar results were obtained when firefly luciferase was coexpressed. The activity of this enzyme found in extracts from cells cotransfected with pwtGH and either of the expression plasmids for the deletion mutants del32-69 or del32-71 was approximately 5-fold lower in comparison to the pwtGH/pwtGH control transfection (Fig. 13) and the degree of inhibition was also dose-dependent (data not shown).

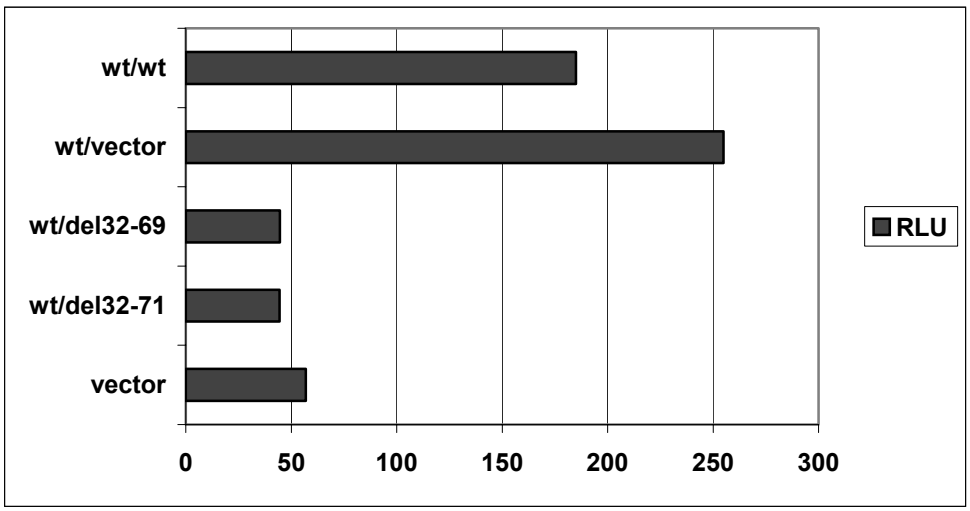

Fig. 13

Firefly luciferase activities in cell lysates. Cells were always cotransfected with 0.4 µg of each of the plasmids denoted and 0.2 µg of plasmid pGL2LUC. Values of a single transfection are presented. Luciferase activity was severely diminished in the presence of del32-71GH.

In order to investigate if the observed effects on heterologous proteins were independent of the presence of wtGH protein in addition to the mutant GH protein, or if a molecular interaction between both types of GH proteins is a prerequisite for these phenomena, the luciferase activity was measured in cells cotransfected only with pdel32-71GH and pGL2LUC. In this case, luciferase activity was barely detectable and comparable to that detected in extracts of untransfected cells, while those cotransfected with pwtGH and pGL2LUC yielded considerable luciferase activities (data not shown). Therefore, the effect of del32-71GH on heterologously coexpressed proteins was independent on the presence of wtGH.

Analogous results were obtained when IGFBP-2 levels were determined in culture media of cells which were cotransfected with pwtGH, either of the expression plasmids for a GH mutant, and pIGFBP-2. The concentrations of IGFBP-2 detected in media were inversely correlated to the extent of the deletion greater than 32-52 in the transfected mutant constructs. Cotransfection of either of the point mutants showed no significant effect on IGFBP-2 amounts (Fig. 14).

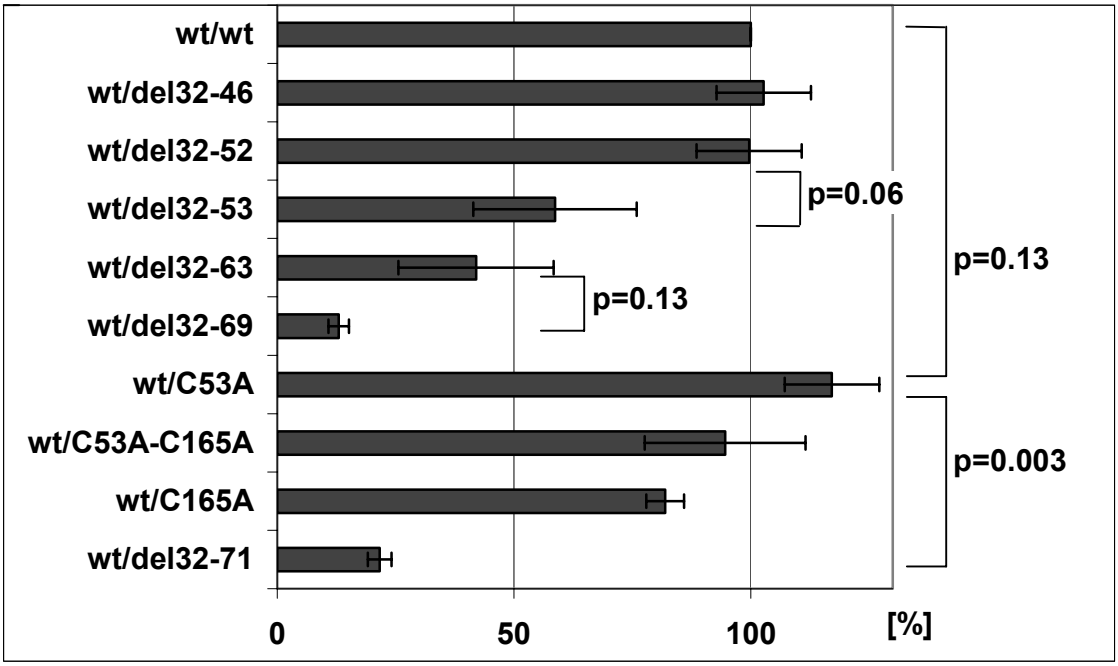

Figure 14

IGFBP-2 concentrations measured using RIA in media of cells cotransfected with pIGFBP-2, pwtGH and plasmids encoding GH deletion or point mutants. Mean values of 3 individual experiments +/-SD are represented as per cent of the values obtained for the pwtGH/pwtGH transfection which was set to 100%. The IGFBP-2 concentration in each sample was measured twice, using the mean value for calculations. P-values are given for the indicated comparisons.

Real-time RT-PCR results revealed comparable mRNA expression levels for wtGH as well as for total hGH and for the marker protein IGFBP-2 (Fig. 15) indicating that the observations on the protein level were not based on decrease of transcription or RNA instability.

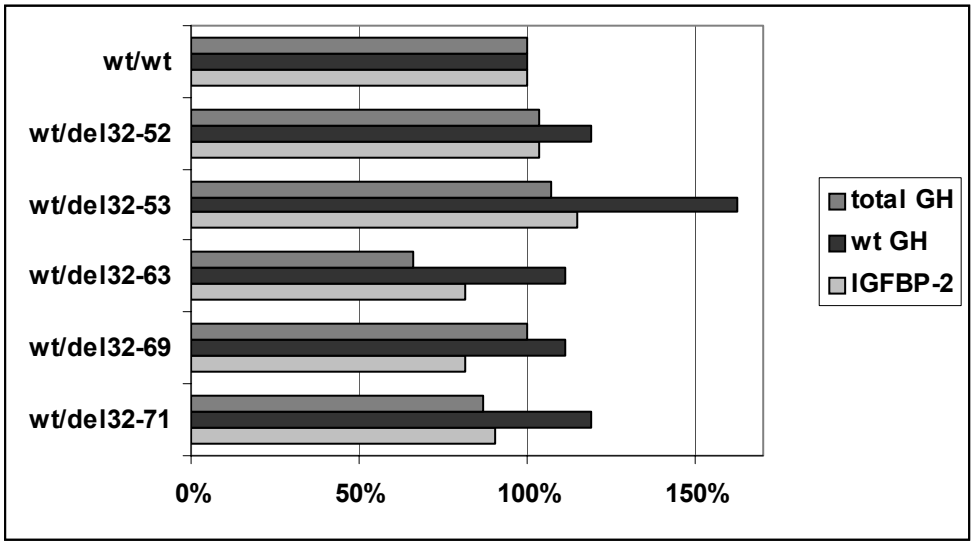

Figure 15

Relative expression level of wtGH, total GH, and IGFBP-2 mRNA for cells cotransfected with pwtGH, plasmids encoding GH deletion mutants, and pIGFBP-2. Data are represented as per cent of the values obtained for the pwtGH/pwtGH transfection which was set to 100%. For each sample, duplicate measurements were performed, using the mean value for calculations.

7. Discussion

The presence of specific GH protein mutants in the pituitary somatotrophs results in a dominant restriction of wtGH secretion while others are tolerated at the heterozygous state. Since the expression of specific GH protein mutants results in dominantly transmitted phenotypes while others do not affect wtGH secretion, it was of great interest to analyse in further details the effects of different GH protein structures on dimerization/aggregation of mutant and wt protein at some point of the secretory pathway and on the degradation and disposal of misfolded mutant proteins by the cells degradation systems. Therefore, it was of importance to analyze the relationship between *GH-1* mutations and effects as well as the kind and extent of mutations associated with major or minor effects in further detail. The focus was set on studying the role of the linker region between helices 1 and 2, the deletion of which is frequently the cause of IGHD II. This region contains a cysteine at position 53 that is necessary for the formation of an intramolecular disulfide bridge with Cys165. The data of previous studies have suggested that the presence of free cysteine residues, which can potentially form intermolecular disulfide bridges, has only a minor influence on the exhibition of the dominant-negative effect. The deletion mutant del32-71,C165A-GH bearing an exchange of cysteine to alanine at position 165 in addition to the deletion of aa 32-71 exerted a dominant-negative effect on wtGH secretion in a similar manner as del32-71GH (41). Our aim, however, was to investigate the properties of constructs bearing no deletions but only point mutations. Mutation of one cysteine residue at positions 53 or 165 eliminated the formation of the intramolecular disulfide bridge Cys53-Cys165 and made the establishment of irregular intramolecular – with cysteine residues at positions 182 or 189 – or intermolecular disulfide bridges possible (Fig. 1). The last mechanism would potentially facilitate the formation of GH molecule dimers covalently bound to each other. Mutations of both cysteine residues at positions 53 and 165 eliminated the formation of disulfide bridges totally. Our results suggest that disruption of the disulfide bridge alone is not

sufficient for the exhibition of the dominant-negative effect since the GH concentration in cells cotransfected with pwtGH and an expression plasmid for either of the point mutants was only slightly reduced. In addition, the point mutants with one free cysteine (C53A or C165A) displayed similar behaviour in comparison to the mutant harbouring two aa exchanges (C53A-C165A). Our results are in line with those of a previous study suggesting that the presence of the free cysteine residue 165 has only a minor influence on the exhibition of the dominant-negative effect (41). These findings are contradictory to the assumption that del32-71GH affects wtGH secretion only by forming dimers due to free cysteine residues (39) and are not in line with the hypothesis that the presence of an unpaired cysteine residue could explain the exertion of the dominant-negative effect on GH secretion in patients bearing a mutation within exon 5 of *GH-1* which disrupts the disulfide bridge Cys182-Cys189 (34).

Our construct del32-52GH was related to the naturally-occurring 20 kD GH variant (del32-46) lacking additional six aa but preserving the cysteine at position 53 and, therefore, the intramolecular disulfide bridge. The concentration of GH detected both in media and cell extracts of cells cotransfected with pwtGH and either pdel32-46GH or pdel32-52GH was similar. Deletion of only one additional aa in the construct del32-53, thus hampering the formation of the disulfide bridge constituted between aa residues 53 and 165, led to a statistically significant decrease ($p=0.006$) of the concentration of secreted GH in comparison to del32-52GH. Moreover, the inhibitory effect of del32-53GH on wtGH secretion was shown to be dose-dependent. This observation indicates that disruption of the disulfide bridge is a step-change event. This disruption evidently affects the tertiary structure of the GH molecule critically when residue 53 is removed in the context of the whole deleted aa stretch 32-53; thus, the relationship between structure and function is not simply linearly correlated to the extent of the peptide deletion. Deletion of ten additional aa up to the beginning of the second minihelix (Fig. 1) in construct del32-63GH induced no further reduction of the hGH detected,

while the additional deletion of the second minihelix encompassing six supplementary aa in del32-69GH resulted in a negative effect comparable to that of del32-71GH.

One hypothesis for the exhibition of the dominant-negative effect exerted by some GH mutants explains the decreased secretion with a disorder of the aggregation of GH molecules. It has been suggested that the binding of Zn^{2+} by GH molecules through amino acid residues His18, His21 and Glu174 is a prerequisite for GH dimerization and subsequently for GH storage in secretory granules (52). Since deletion of the linker region between alpha-helices 1 and 2 presumably results in a strongly changed tertiary structure of the GH molecule, this might also affect Zn^{2+}-binding and as a consequence influence aggregation or sorting of GH proteins, or the maturation of the secretory granules (53). Although our cotransfecton experiments did not reveal any dominant-negative effect exerted by the point mutants which are supposed to bind zinc poorly or not at all, an increase in the extent of the deletion between the first and the second alpha-helices and, in particular, deletion of pronounced structures like the second minihelix is likely to result in major structural changes and consequently strong disturbances of aggregation.

GH immunoreactivity assayed using a polyclonal RIA was the main parameter studied in our *in-vitro* experiments. The polyclonal antiserum of this RIA has a presumed wide spectrum of epitope specificities (27). It has been well characterized for the determination of 22 kD GH (50), but its crossreactivity to mutant GH proteins, especially del32-71GH (17.5 kD GH) is not known. Therefore, there is no doubt that in those experiments where wtGH was expressed using the same amounts of pwtGH a *substantial decrease* of RIA values did represent a decrease of 22 kD GH. In experiments with *minor decrease* of RIA values, the change could be caused by a decrease of GH amounts or by decreased crossreactivity or a combination of both from a theoretical standpoint. However, our data of Western blot analysis which

avoided the direct competition between wt and mutant GH correlate well with RIA values. In addition, Western blot analysis using a monoclonal antibody raised against an epitope not affected by the GH mutations confirmed this finding. These experimental data strongly argue in favour of that immunoreactivity measured using the polyclonal RIA reflects indeed total GH amounts rather than lack of GH crossreactivity. Therefore, these results underline the usefulness of the applied GH RIA for the detection of the different GH mutants.

GH RIA results are presented without correction. However, transfection efficiency determined by counting β-galactosidase stained cells was equal. The discrepancy with the luminometric data could be due to different expression at the protein level despite the equivalent efficiency of transfection as the intensity of staining observed under the microscope decreased in the presence of del32-71GH. In addition, the absence of significant differences of expression at the mRNA level of total GH and wtGH were also indicative for a comparable transfection efficiency.

Toxic effects in cells cotransfected with pwtGH and pdel32-71GH were not found on the basis of a cell viability analysis. There was no decrease in cell numbers 80 hours post-transfection which assumably would have been the case if del32-71GH exerted cell toxicity despite the relatively low percentage of the cell population being transfected. In addition, mRNA expression of the marker protein IGFBP-2 was unaffected by coexpression of del32-71GH. These results are in agreement with an earlier report in which lack of toxicity over a 24-hour-period was suggested by the observation that the secretory pathway in GH_4C_1 cells expressing del32-71GH functioned properly (41). It has to be emphasized that our data were obtained over a relatively short period of time. Therefore, we cannot exclude effects on cell viability due to inhibition of non-GH protein production over a longer term.

The observed severe negative effect of del32-69GH and del32-71GH not only on wtGH but also on IGFBP-2 secretion as well as on β-galactosidase and firefly luciferase activities suggests a general disturbance of cells expressing mutant GH beyond the molecular interaction of wtGH and mutant GH. Also, β-galactosidase and luciferase are not expected to be transported through the regulated secretory pathway. Our findings are not in line with experiments applying the neuroendocrine cell lines MtT/S and AtT-20 where del32-71GH had no clear decreasing effect on β-galactosidase activity (40). However, in the experimental setting cited the effect of del32-71GH on wtGH secretion was much less pronounced requiring a high excess of mutant GH for an observable effect, thereby making the missing effect on β-galactosidase less significant. These divergent data may be explained by different transfection conditions. In addition, some effects of del32-69GH and del32-71GH may be cell-type specific; GH_4C_1, a rat prolactin and GH producing cell line, is a somatotroph-derived cell line while AtT-20 is adrenocorticotroph-derived.

Recently McGuinness *et al.* reported that rat pituitary GC cells, which were stably mono-transfected with a plasmid expressing human del32-71GH, showed a decreased proliferation rate, reduced attachment and disturbed morphology followed by an early cell apoptosis, thus indicating toxic-like effects of del32-71GH on this cell line (45). This discrepancy to our data may be explained by cell-specificities since GH_4C_1 cells stably cotransfected with pwtGH and pdel32-71GH exhibit viability and proliferation rates similar to those of cells mono-transfected with pwtGH (data not shown). Therefore, every cell system may potentially react in a specific manner to the mutant GH expression, possibly different from what human somatotrophs would show *in vitro*. However, we believe that our cell system is a good model for studying specific features of mutant human GH as GH_4C_1 cells are neuroendocrine somatotroph cells.

The phenomena of suppression of wtGH secretion and the negative effect exerted on the other proteins discussed presumably share a common

genesis. As earlier postulated, all steps from translation, posttranslational modification, protein storage and secretion could possibly be involved in the origination of the dominant negative effect (39). The presence of the deletion mutant possibly disturbs various biological functions of cells without affecting their viability and proliferation rate. The expression of misfolded proteins is a source of cell stress which results in degradation of proteins due to activation of unfolded protein responses (54). Presumably, the level of heterologous proteins is affected to a higher degree in comparison to endogenous proteins because the former are produced in higher quantities and are thus more prone to be affected by degradation systems induced by unfolded protein responses.

Our data suggest that – besides a minor role of the integrity of the disulfide bridge Cys53-Cys165 – the extent of deletion plays the major role in expression of the dominant-negative effect. The inhibitory effect of GH mutants on heterologously expressed non-GH proteins suggests that the dominant-negative effect is not limited to GH and not even to proteins of the regulated secretory pathway but may depend on expression levels.

8. Acknowledgements

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9. Summary

Different mutant forms of GH display different behaviours, and hence they lead to different clinical consequences. Monogenetic isolated GHD can be divided into types I, II, and III. IGHD I is transmitted in an autosomal recessive way and is further subdivided into IGHD IA and IGHD IB. Patients with IGHD IA have a complete lack of GH in the circulation as they bear a homozygous deletion of *GH-1*, the gene encoding hGH. Treatment of these patients is difficult and almost always not successful, as they develop antibodies against rhGH. Different genetic backgrounds can be the reason for the most common type of inherited IGHD – type IB. In few patients splice site mutations in intron IV of *GH-1* resulting in skipping of the nucleotides coding for amino acids 103 to 126 and subsequently in a frame shift in the exon 5 encoded region, or inactivating mutations of the gene encoding the growth hormone releasing hormone receptor were found. X-linked transmitting is typical for the rare IGHD III.

Isolated growth hormone deficiency type II is transmitted in an autosomal dominant manner. It is a relatively rare entity, the aetiology of which is known but the subtle pathogenetic mechanisms are unclear. It is associated with a severely insufficient GH output and as a result affected patients have growth failure starting early after birth, proportionate short stature, acromicria, truncal fat deposition, high-pitched voice, delayed bone age. An excellent response to substitutive therapy with rhGH is typical for this disorder.

IGHD II is mainly caused by monoallelic splice site mutations in intron 3 of *GH-1* leading to skipping of exon 3 which encodes amino acids 32-71, the connecting loop between the alpha-helices 1 and 2 in the hGH molecule. The GH mutant protein is thus subject to serious structural changes. In patients harbouring this kind of mutation GH concentrations are extremely low even though one intact *GH-1* allele is present. The production of the resulting mutant protein del32-71GH causes a blockade of wild type GH secretion by a

still obscure mechanism. In addition, point mutations affecting highly conserved amino-acids situated at protruding sites of the hGH molecule can cause IGHD II. To date, the following point mutations have been described as a cause of dominantly transmitted IGHD – P89L-GH, V110F-GH, and R183H-GH.

Several hypotheses have been discussed towards explaining the basic mechanisms of the interference of some mutant GH forms with wtGH. These include (i) accumulation of toxic aggregates of mutant proteins; (ii) decrease of intracellular stability of wtGH due to cellular responses induced by unfolded proteins (overload response, unfolded protein response, induction of cell type specific degradation systems); (iii) a specific blockade of GH aggregation and/or sorting into secretory granules; and (iv) impaired maturation of secretory granules.

The aim of this *in-vitro* study using GH_4C_1 cells was to elucidate the importance of specific amino acids or stretches of amino acids in the context of the GH tertiary structure for the exhibition of the dominant-negative effect.

In order to investigate the structure-function relationship of hGH mutants with respect to a negative effect on wtGH secretion, a series of hGH constructs which were mutated or deleted in specific amino acids or stretches of amino acids was generated. The focus was set on the study of the linker region between alpha-helices 1 and 2 and especially between aa 46 and 71 since del32-46GH (the 20 kD GH variant form) exerts no deleterious effect while del32-71GH has a strong adverse effect on wtGH secretion. Therefore, diverse deletion mutants, del32-46, del32-52, del32-53, del32-63, and del32-69 were constructed in which the respective amino-acid stretch was deleted. Of additional interest was the role of the disulfide bridge constituted between Cys53 and Cys165 which is disrupted in del32-71GH. Distinct point mutants were constructed in which the respective cysteine encoding DNA triplets were

mutated to alanine encoding triplets, resulting in the GH mutants C53A, C165A and C53A-C165A.

The role of the disulfide bridge constituted between Cys182 and Cys189 was also of interest for the exertion of a potential dominant-negative effect. Therefore, the deletion mutant del188-190GH was constructed in which the respective amino-acid stretch was deleted.

The relevance of Zn^{2+}-binding of the hGH molecule for the production, processing, sorting into secretory granules, and secretion of GH was also studied. For this purpose a series of point mutants in which the triplets encoding the amino-acid histidine at positions 18 and 21, as well as triplets encoding the amino-acid glutamine at position 174 were mutated to triplets encoding the amino-acid alanine, thus preventing partially or completely Zn^{2+}-binding. The mutants were as follows: H18A, H21A, G174A, H18A-G174A, H21A-G174A, H18A-H21A, and H18A-H21A-G174A. In these mutants one, two or all three Zn^{2+}-binding amino-acids were substituted by the amino-acid alanine which does not bind Zn^{2+}. GH_4C_1 cells were transiently transfected with the constructs and the amount of hGH was estimated by a RIA using polyclonal anti hGH antiserum.

The point mutants with an isolated disruption of the disulfide bridge Cys53-Cys165 exerted no dominant-negative effect on the secretion of wtGH. In comparison to the control transfection only with pwtGH, the amount of hGH secreted into the medium was only mildly decreased by 15-30% when coexpressing wtGH and either of the point mutants C53A, C165A or C53A-C165A. Concerning the deletion mutants, the degree of GH reduction was proportional to the increase in size of the deletion. Coexpression of wtGH and mutants deleted in amino acids 32-46 or 32-52 resulted in a mild reduction of the amount of hGH detected in media. Deletion of more amino acids (32-53, 32-63) led to a stronger decrease of secreted hGH by 50-60%. This observation indicates that disruption of the disulfide bridge is a step-change

event. This disruption evidently affects the tertiary structure of the GH molecule critically when residue 53 is removed in the context of the whole deleted aa stretch 32-53; thus, the relationship between structure and function is not simply linearly correlated to the extent of the peptide deletion. A severe decrease by 75-85% was observed when stretches 32-69 or 32-71 were deleted. The same relationship between the extent of deletion and the amount of detectable hGH was found in protein extracts of the respectively transfected cells. The extent of deletion in combination with the loss of the disulfide bridge played a role in the expression of the dominant-negative effect.

This negative effect was observed not merely limited to the secretion of GH. A similar inverse correlation was found between the extent of deletion of the hGH molecule and the amount/activity of three different marker proteins – beta-galactosidase, firefly luciferase, and IGFBP-2. Real-time RT-PCR results revealed comparable mRNA expression levels for wtGH as well as for total hGH and for the marker protein IGFBP-2, a finding which indicates that the observations on the protein level were not based on decrease of transcription or RNA instability.

The postulated important role of the second disulfide bridge constituted between Cys182 and Cys189 was not confirmed in our cell model system. Its disruption was not connected with any significant effect on the wtGH production and secretion.

The role of Zn^{2+}-binding of hGH molecule was not proven to be crucial for the exertion of the dominant-negative effect. The working hypothesis of a slight effect exerted by the mono-mutants, a moderate effect exerted by the mutants with two aa exchanges and a strong dominant-negative effect exerted by the mutant with three aa exchanges (a mutant which does not bind Zn^{2+}) was not confirmed in our cell model system.

In order to prove if the amount of hGH was underestimated in the RIA due to the intrinsic competition with recombinant hGH, cell extracts and media were analyzed using Western blots. Densitometric evaluation of protein bands corresponding to wtGH and mutant GH (detected by polyclonal antiserum as well as by monoclonal antibodies directed towards the N-terminal domain of hGH) revealed comparable amounts as determined using RIA, and thus proved the usefulness of the applied RIA.

The total cell numbers and the viability of cells expressing either only wtGH or coexpressing wtGH and del32-71GH stayed equivalent in individual experiments over periods of up to 80 hours, thus making an acute toxic effect of del32-71GH on the cells unlikely.

The transfection efficiency was determined in individual experiments by counting the beta-galactosidase stained cells directly on culture dish and was found to be between 10 and 15%. No intraexperimental variation of transfection efficiency was found between cells transfected only with pwtGH or those cotransfected with pwtGH and either pdel32-46, pdel32-53, or pdel32-71GH. Interestingly, the intensity of the staining decreased with the increasing length of the deleted stretch. GH RIA results are presented without correction as transfection efficiency was equal. In addition, the absence of significant differences of expression at the mRNA level of total GH and wtGH were also indicative for a comparable transfection efficiency.

These data suggest that – besides a minor role of the integrity of the disulfide bridge Cys53-Cys165 – the extent of deletion in the studied region plays the major role in expression of the dominant-negative effect. The inhibitory effect of GH mutants on heterologously expressed non-GH proteins suggests that the dominant-negative effect is not limited to GH and not even to proteins of the regulated secretory pathway but may depend on expression levels.

10. References

1. **Evans HM, Long JA** 1921 The effect of the anterior lobe administered intraperitoneally upon growth, maturity and oestrus cycles of the rat. Anat Rec 21: 62-63

2. **Li CH, Evans HM** 1944 The isolation of pituitary growth hormone. Science 99: 183-184

3. **Soyka LF** 1966 The problem of short stature in childhood: growth hormone deficiency and its treatment with human growth hormone. Med Times 94(1): 1-8

4. **Sheikholislam BM, Stempfel RS Jr** 1972 Hereditary isolated somatotropin deficiency: effects of human growth hormone administration. Pediatrics 49(3): 362-374

5. **Tanner JM, Whitehouse RH, Hughes PC, Vince FP** 1971 Effect of human growth hormone treatment for 1 to 7 years on growth of 100 children, with growth hormone deficiency, low birthweight, inherited smallness, Turner's syndrome, and other complaints. Arch Dis Child 46(250): 745-782

6. **Poskitt EM, Rayner PH** 1974 Isolated growth hormone deficiency. Two families with autosomal dominant inheritance. Arch Dis Child 49(1): 55-59

7. **van Gelderen HH, van der Hoog CE** 1981 Familial isolated growth hormone deficiency. Clin Genet 20(2): 173-175

8. **Dattani MT** 2004 Novel insights into the aetiology and pathogenesis of hypopituitarism. Horm Res 62, Suppl. 3: 1-13

9. **Tatsumi K, Miyai K, Notomi T, Kaibe K, Amino N, Mizuno Y, Kohno H** 1992 Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene. Nat Genet 1(1): 56-58

10. **Wu W, Cogan JD, Pfaffle RW, Dasen JS, Frisch H, O'Connell SM, Flynn SE, Brown MR, Mullis PE, Parks JS, Phillips JA 3rd, Rosenfeld MG** 1998 Mutations in PROP1 cause familial combined pituitary hormone deficiency. Nat Genet 18(2): 147-149

11. **Cogan JD, Wu W, Phillips JA 3rd, Arnhold IJ, Agapito A, Fofanova OV, Osorio MG, Bircan I, Moreno A, Mendonca BB** 1998 The PROP1 2-base pair deletion is a common cause of combined pituitary hormone deficiency. J Clin Endocrinol Metab 83(9): 3346-3349

12. **Netchine I, Sobrier ML, Krude H, Schnabel D, Maghnie M, Marcos E, Duriez B, Cacheux V, Moers A, Goossens M, Gruters A, Amselem S** 2000 Mutations in LHX3 result in a new syndrome revealed by combined pituitary hormone deficiency. Nat Genet 25(2): 182-186

13. **Dattani MT, Martinez-Barbera JP, Thomas PQ, Brickman JM, Gupta R, Martensson IL, Toresson H, Fox M, Wales JK, Hindmarsh PC, Krauss S, Beddington RS, Robinson IC** 1998 Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. Nat Genet 19(2): 125-133

14. **Vimpani GV, Vimpani AF, Lidgard GP, Cameron EH, Farquhar JW** 1977 Prevalence of severe growth hormone deficiency. Br Med J 2(6084): 427-430

15. **Phillips JA, Cogan JD** 1994 Genetic basis of endocrine disease. 6. Molecular basis of familial human growth hormone deficiency. J Clin Endocrinol Metab 78:11-16

16. **Phillips JA 3rd, Hjelle BL, Seeburg PH, Zachmann M** 1981 Molecular basis for familial isolated growth hormone deficiency. Proc Natl Acad Sci USA 78:6372-6375

17. **Akinci A, Kanaka C, Eble A, Akar N, Vidinlisan S, Mullis PE** 1992 Isolated growth hormone (GH) deficiency type IA associated with a 45-kilobase gene deletion within the human GH gene cluster. J Clin Endocrinol Metab 75:437-441

18. **Procter AM, Phillips JA, Cooper DN** 1998 The molecular genetics of growth hormone deficiency. Hum Genet 103:255-272

19. **Baumann G, Maheshwari H** 1997 The Dwarfs of Sindh: severe growth hormone (GH) deficiency caused by a mutation in the GH-releasing hormone receptor gene. Acta Paediatr Suppl 423:33-38

20. **Binder G, Ranke MB** 1995 Screening for growth hormone (GH) gene splice-site mutations in sporadic cases with severe isolated GH deficiency using ectopic transcript analysis. J Clin Endocrinol Metab 80:1247-1252

21. **Cogan JD, Phillips JA, Schenkman SS, Milner RD, Sakati N** 1994 Familial growth hormone deficiency: a model of dominant and recessive mutations affecting a monomeric protein. J Clin Endocrinol Metab 79:1261-1265

22. **Cogan JD, Ramel B, Lehto M, Phillips J 3rd, Prince M, Blizzard RM, de Ravel TJ, Brammert M, Groop L** 1995 A recurring dominant

negative mutation causes autosomal dominant growth hormone deficiency--a clinical research center study. J Clin Endocrinol Metab 80(12): 3591-3595

23. **Massa GG, Binder G, Oostdijk W, Ranke MB, Wit JM** 1998 De novo mutations of the growth hormone gene: an important cause of congenital isolated growth hormone deficiency? Eur J Pediatr 157(4): 272-275

24. **Ultsch MH, Somers W, Kossiakoff AA, de Vos AM** 1994 The crystal structure of affinity-matured human growth hormone at 2 A resolution. J Mol Biol 236:286-299

25. **Mullis PE, Deladoey J, Dannies PS** 2002 New GH-1 gene mutations: expanding the spectrum of causes of isolated growth hormone deficiency. J Pediatr Endocrinol Metab 15 Suppl 5: 1301-1310

26. **Mullis PE, Deladoey J, Dannies PS** 2002 Molecular and cellular basis of isolated dominant-negative growth hormone deficiency, IGHD type II: insights on the secretory pathway of peptide hormones. Horm Res 58(2): 53-66

27. **Baumann G** 1991 Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endocr Rev 12:424-449

28. **Binder G, Keller E, Mix M, Massa GG, Stokvis-Brantsma WH, Wit JM, Ranke MB** 2001 Isolated GH deficiency with dominant inheritance: new mutations, new insights. J Clin Endocrinol Metab 86(8): 3877-3881.

29. **Gertner JM, Wajnrajch MP, Leibel RL** 1998 Genetic defects in the control of growth hormone secretion. Horm Res.; 49 Suppl 9-14.

30. **Duquesnov P, Simon D, Netchine I, Dastot F, Sobrier ML, Goossens M, Czernichow P, Amselem S** 1998 Familial isolated growth hormone deficiency with slight height reduction due to a heterozygote mutation in GH gene. 80th Ann. Meet., Endocr. Soc., Abstr. P2-202

31. **Deladoey J, Stocker P, Mullis PE** 2001 Autosomal dominant GH deficiency due to an Arg183His GH-1 gene mutation: clinical and molecular evidence of impaired regulated GH secretion. J Clin Endocrinol Metab. 86: 3941-3947

32. **Igarashi Y, Ogawa M, Kamijo T, Iwatani N, Nishi Y, Kohno H, Masumura T, Koga J** 1993 A new mutation causing inherited growth hormone deficiency: a compound heterozygote of a 6.7 kb deletion and a two base deletion in the third exon of the GH-1 gene. Hum Mol Genet 2: 1073-1074

33. **Takahashi Y, Kaji H, Okimura Y, Goji K, Abe H, Chihara K** 1996 Brief report: short stature caused by a mutant growth hormone. N Engl J Med 334(7): 432-436

34. **Weigel JFW, Hesse V, Stobbe HM, Ulmen U, Kiess W, Pfaeffle RW** 2004 Familial isolated growth hormone deficiency type II; a new mutation within exon 5 supporting evidence that an unpaired cystein exerts the dominant-negative effect on GH secretion. 86th Ann. Meet., Endocr. Soc., Abstr. P3-385

35. **Cooke NE, Liebhaber SA** 1995 Molecular biology of the growth hormone-prolactin gene system. Vitam Horm 50: 385-459

36. **Sun Z, Lee MS, Rhee HK, Arrandale JM, Dannies PS** 1997 Inefficient secretion of human H27A-prolactin, a mutant that does not bind Zn2+. Mol Endocrinol 11: 1544-1551

37. **Yang TH, Cleland JL, Lam X, Meyer JD, Jones LS, Randolph TW, Manning MC, Carpenter JF** 2000 Effect of zinc binding and precipitation on structures of recombinant human growth hormone and nerve growth factor. J Pharm Sci 89: 1480-1485

38. **Duda KM, Brooks CL** 2003 Differential effects of zinc on functionally distinct human growth hormone mutations. Protein Eng 16: 531-534

39. **Binder G, Brown M, Parks JS** 1996 Mechanisms responsible for dominant expression of human growth hormone gene mutations. J Clin Endocrinol Metab 81:4047-4050

40. **Hayashi Y, Yamamoto M, Ohmori S, Kamijo T, Ogawa M, Seo H** 1999 Inhibition of growth hormone (GH) secretion by a mutant GH-I gene product in neuroendocrine cells containing secretory granules: an implication for isolated GH deficiency inherited in an autosomal dominant manner. J Clin Endocrinol Metab 84:2134-2139

41. **Lee MS, Wajnrajch MP, Kim SS, Plotnick LP, Wang J, Gertner JM, Leibel RL, Dannies PS** 2000 Autosomal dominant growth hormone (GH) deficiency type II: the del32-71-GH deletion mutant suppresses secretion of wild-type GH. Endocrinology 141:883-890

42. **Dannies PS** 2000 Protein folding and deficiencies caused by dominant-negative mutants of hormones. Vitam Horm 58:1-26

43. **Kim PS, Arvan P** 1998 Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: disorders of protein trafficking and the role of ER molecular chaperones. Endocr Rev 19(2): 173-202

44. **Graves TK, Patel S, Dannies PS, Hinkle PM** 2001 Misfolded growth hormone causes fragmentation of the Golgi apparatus and disrupts endoplasmic reticulum-to-Golgi traffic. J Cell Sci 114(20):3685-3694

45. **McGuinness L, Magoulas C, Sesay AK, Mathers K, Carmignac D, Manneville JB, Christian H, Phillips JA 3rd, Robinson IC** 2003 Autosomal dominant growth hormone deficiency disrupts secretory vesicles in vitro and in vivo in transgenic mice. Endocrinology 144:720-731

46. **Binder G, Nagel BH, Ranke MB, Mullis PE** 2002 Isolated GH deficiency (IGHD) type II: imaging of the pituitary gland by magnetic resonance reveals characteristic differences in comparison with severe IGHD of unknown origin. Eur J Endocrinol 147:755-760

47. **Dannies PS** 2000 Protein folding and deficiencies caused by dominant-negative mutants of hormones. Vitam Horm 58: 1-26

48. **Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR** 1989 Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51-59

49. **Chen EY, Liao YC, Smith DH, Barrera-Saldana HA, Gelinas RE, Seeburg PH** 1989 The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics 4(4): 479-497

50. **Hauffa BP, Lehmann N, Bettendorf M, Mehls O, Dorr HG, Partsch CJ, Schwarz HP, Stahnke N, Steinkamp H, Said E, Sander S,**

Ranke MB 2004 Central reassessment of GH concentrations measured at local treatment centers in children with impaired growth: consequences for patient management. Eur J Endocrinol 150:291-297

51. **Elmlinger MW, Wimmer K, Biemer E, Blum WF, Ranke MB Dannecker GE** 1996 Insulin-like growth factor binding protein 2 is differentially expressed in leukaemic B- and T-cell lines. Growth Regul 6:152-157

52. **Cunningham BC, Mulkerrin MG, Wells JA** 1991 Dimerization of human growth hormone by zinc. Science 253(5019): 545-548

53. **Moore HH, Kelly RB** 1986 Re-routing of a secretory protein by fusion with human growth hormone sequences. Nature 321(6068): 443-446

54. **Kauffman KJ, Pridgen EM, Doyle FJ 3rd, Dhurjati PS, Robinson AS** 2002 Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in Saccharomyces cerevisiae. Biotechnol Prog 18:942-950

11. Curriculum vitae

PERSÖNLICHE DATEN:

Name:	Daniel Iliev
Geburtsdatum:	31.05.1970
Geburtsort:	Sofia, Bulgarien
Nationalität:	bulgarisch
Mutter:	Snejina Manolova, Chemikerin
Vater:	Ilia Manolov, Chemiker
Dienstadresse:	Universitätskinderklinik Abteilung Endokrinologie Hoppe-Seyler-Str. 1 D-72076 Tübingen
Telefonnummer:	07071 29 81335 oder 29 85767
FAX:	07071 29 4157
Email:	Daniel.Iliev@med.uni-tuebingen.de
Familienstand:	verheiratet, 1 Kind
Ehefrau:	Tanja Stoilkova-Ilieva, Chemikerin
Kind:	Snejina Ilieva, geb. am 05.06.2000
BILDUNG:	Grundschule und Gymnasium “Elin Pelin”, Sofia, 1977-1984; Gymnasium mit sprachlichem Zug (Englische Sprachschule), Sofia, 1984-1989 Abitur, 1989 Medizinstudium an der Universität Sofia, 1989-1995; Staatsexamina, 1995 Mai 2002 Facharzt für Pädiatrie
SONSTIGES:	„ESPE Winter School“, 1998, Sofia, Bulgarien „ESPE Summer School“, 2000, Castel les Sorbiers, Belgien

BERUFSERFAHRUNG UND DERZEITIGE TÄTIGKEIT:	
März 1996 – Oktober 1999	Assistenzarzt Universitätskinderklinik Sofia Abteilung Endokrinologie and Diabetologie
November 1999 – November 2000	ESPE Stipendiat für Pädiatrische Endokrinologie bei Prof. M.B. Ranke, Universitätskinderklinik Tübingen
November 2000 – Juni 2002	Assistenzarzt, Universitätskinderklinik Sofia Abteilung Endokrinologie
Juni 2002 – Januar 2003	Facharzt, Universitätskinderklinik Sofia Abteilung Endokrinologie
Seit Januar 2003	ESPE Forschung-Stipendiat für 2 Jahre für Pädiatrische Endokrinologie bei Prof. Dr. M.B. Ranke und PD Dr. G. Binder Universitätskinderklinik Tübingen
21.01.2005	Mündliche Doktorprüfung
FORSCHUNGSBEREICHE:	Isolierter Wachstumshormon-Mangel Typ II: Mechanismen dominant-negativer Mutationen; Aromatase-Gen und präpubertäre Gynäkomastie
BERUFSZIEL:	Pädiatrie, Pädiatrische Endokrinologie
FREMDSPRACHEN:	
Deutsch:	gute Kenntnisse
Englisch:	sehr gute Kenntnisse
Russisch:	gute Kenntnisse