In Vitro Characterization of Human Growth Hormone Mutants

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von

M.Sc. Riia Karoliina Junnila

Präsident der Humboldt-Universität zu Berlin:

Prof. Dr. Dr. h.c. Cristoph Markschies

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I:

Prof. Dr. Andreas Herrmann

Gutachter/in:

1. Prof. Dr. Gudrun Brockmann
2. Prof. Dr. Werner Kloas
3. Prof. Dr. Christian Strasburger

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Abstract

Growth hormone (GH) is a 22 kD, 191-aa, pituitary-derived peptide hormone that is essential for postnatal growth. GH signals via binding to GH receptor (GHR), which initiates intracellular signal transduction pathways. This leads to activation of target genes, most importantly the one encoding insulin-like growth factor (IGF)-1, which mediates most GH action.

In this study a novel GH mutant, Δ188-190, was characterized along with previously reported GH mutants R77C and D112G. All of these mutants had been identified in heterozygous form in patients with retarded growth. Based on patient data, Δ188-190 was thought to be a GHR antagonist. Moreover, the extremely conserved C-terminal disulfide bridge of GH was disrupted in mutant Δ188-190 and its role was studied by substituting one or both of the involved cysteines with alanines.

All mutants and wild type (wt) GH were produced in human embryonic kidney (HEK)-293 cells and an array of *in vitro* experiments was established for their characterization. It turned out that the novel Δ188-190 mutant is not a GHR antagonist after all. It has a diminished binding affinity to GHR, low biological activity and high stability compared to wt GH. R77C and D112G are rather similar to wt GH. The disulfide bridge is important for receptor binding and biological activity of GH. If one of the cysteines is removed the stability of the molecule drops but this can be reversed by removing both cysteines.

If further GH mutants are to be identified, the established array of experiments will be useful for their fast characterization and could even contribute to correct treatment of patients.

growth hormone (GH) – short stature – *GH-1* gene – GHR antagonist – disulfide bridge

Deutsche Zusammenfassung

Wachstumshormon (GH) besteht aus 191 Aminosäuren, hat eine Molekülmasse von 22kD und ist essentiell für postnatales Wachstum. Es wird aus der Adenohypophyse freigesetzt. GH bindet an einen GH-Rezeptor (GHR) und aktiviert somit über intrazelluläre Signalvorgänge Zielgene, insbesondere das, welches für die Kodierung von insulin-like growth factor (IGF-1) zuständig ist. IGF-1 vermittelt den Großteil aller GH-Signale.

Zusammen mit den bereits bekannten GH Mutanten R77C und D112G ist in dieser Studie der neue GH Mutant Δ188-190 charakterisiert worden. Alle drei Mutanten wurden in heterozygoter Form in kleinwüchsigen Patienten identifiziert. Diesen Patientendaten zu Folge schien es möglich, dass Δ188-190 eine GH-antagonistische Wirkung besitzt. Zusätzlich wurde die extrem konservierte C-terminale Disulfidbrücke des GH im Mutanten Δ188-190 unterbrochen vorgefunden. Die Auswirkung der Unterbrechung wurde durch Substitution einer oder beider involvierter Cysteine durch Alanine untersucht.

Alle Mutanten und Wildtypen des GH wurden in menschlichen embryonalen Nierenzellen (HEK-293) angezüchtet und eine Reihe von *in vitro* Experimenten sind für deren Charakterisierung etabliert worden. Es zeigte sich, dass Δ188-190 keine GH-antagonistische Wirkung besitzt. Im Vergleich zum Wildtyp weist der Mutant eine verminderte Bindungsaffinität zu GH, schwächere biologische Aktivität und höhere Stabilität auf. R77C und D112G sind dem Wildtyp GH sehr ähnlich. Die Disulfidbrücke ist wichtig für die Rezeptorbindung und für die biologische Aktivität von GH. Wenn ein Cystein entfernt wird vermindert sich die Stabilität des Moleküls. Dieser Effekt kann durch Entfernen des zweiten Cysteins wieder rückgängig gemacht werden.

Die in dieser Studie etablierten Experimente können Verwendung finden in der Charakterisierung bislang nicht bekannter GH Mutanten und können darüber hinaus zur Behandlung von Patienten eingesetzt werden.

Wachstumshormon (GH) – Kleinwuchs – *GH-1* – GHR Antagonist – Disulfidbrücke

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“Treading their own path, entirely free, are they who are not restrained by fear of failure.”

Sylvi Kekkonen (1900-1974)

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Abbreviations

Δ	deletion
3D	three-dimensional
aa	amino acid
ALS	acid-labile subunit
amp	ampicillin
AMP	adenosine monophosphate
APS	ammonium persulphate
ATP	adenosine triphosphate
b	bovine/ biotin
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
cDNA	complementary DNA
CS	chorionic somatomammotropin (=PL)
CMV	cytomegalovirus
cpm	counts per minute
ddH_2O	double-distilled water
DLR™	Dual-Luciferase® Reporter
D-MEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTPA	diethylene triamine pentaacetic acid
DTT	dithiothreitol
E	exon
EC50	half maximal effective concentration
ECD	extracellular domain
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Eu	europium
FCS	foetal calf serum

FFA	free fatty acid
FPLC	fast protein liquid chromatography
fwd	forward (primer)
GH	growth hormone
GH-1	gene encoding pituitary-derived GH
GHBP	GH binding protein
GH-N	pituitary-derived GH
GHR	GH receptor
GHRH	GH releasing hormone
GHRHR	GHRH receptor
GHS-R1a	growth hormone secretagogue receptor type 1a
GH-V	placental GH
GPCR	G-protein coupled receptor
h	human
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HS	horse serum
IC50	half maximal inhibitory concentration
ICD	intracellular domain
IFA	immunofunctional assay
IFMA	immunofluorometric assay
IGF-1	insulin-like growth factor 1
IGFBP-3	IGF binding protein-3
IgG	immunoglobulin G
IGHD	isolated growth hormone deficiency
IL	interleukin
IRP	international reference preparation
Jak	Janus family tyrosine kinase
kb	Kilobase
kD	kiloDalton
LAR	Luciferase Assay Reagent
LB	lysogeny broth
LCR	locus control region
LHRE	lactogenic hormone responsive element

mAb	monoclonal antibody
MALDI-TOF	matrix assisted laser desorption ionisation-time of flight (MS)
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
mRNA	messenger RNA
MS	mass spectrometry
mut	mutation
ObRb	leptin receptor isoform b
o/d	over day
OD	optical density
o/n	over night
p	pituitary
P	phosphate
PBM	Pharmacy Benefit Manager
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	Protein Data Bank
PDI	protein disulfide isomerase
PEG	polyethylene glycol polymer
PI3K	phosphatidylinositol 3-kinase
Pit-1	pituitary-specific transcription factor-1
PL	placental lactogen
PM	plasma membrane
PRL	prolactin
PRLR	PRL receptor
r	recombinant
RER	rough ER
rev	reverse (primer)
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute (medium)
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SN	supernatant

SOCS	suppressors of cytokine signalling
SST	somatostatin
STAT	signal transducer and activator of transcription
Strep	streptavidin
TEMED	tetramethylethylenediamine
TK	thymidine kinase
TR-IFMA	time-resolved immunofluorometric assay
Tris	Tris-(hydroxymethyl)-aminomethane
Tyk2	tyrosine kinase 2
UTR	untranslated region
wt	wild type

1 Introduction

1.1 Growth hormone

1.1.1 A glance to the past

The most important events in the history of growth hormone are summarised in figure 1. First observation of a growth promoting effect of pituitary extract was reported in the 1920s [Evans, 1922]. It took more than 20 years for the substance to be isolated and designated as pituitary growth hormone [Li, 1944]. Pituitary-derived growth hormone had successfully been used in treatment of pituitary dwarfism already in the 1960s [Prader, 1964; Tanner, 1967] but the expression of growth hormone in bacteria in 1979 took growth hormone therapy to a new level [Martial, 1979].

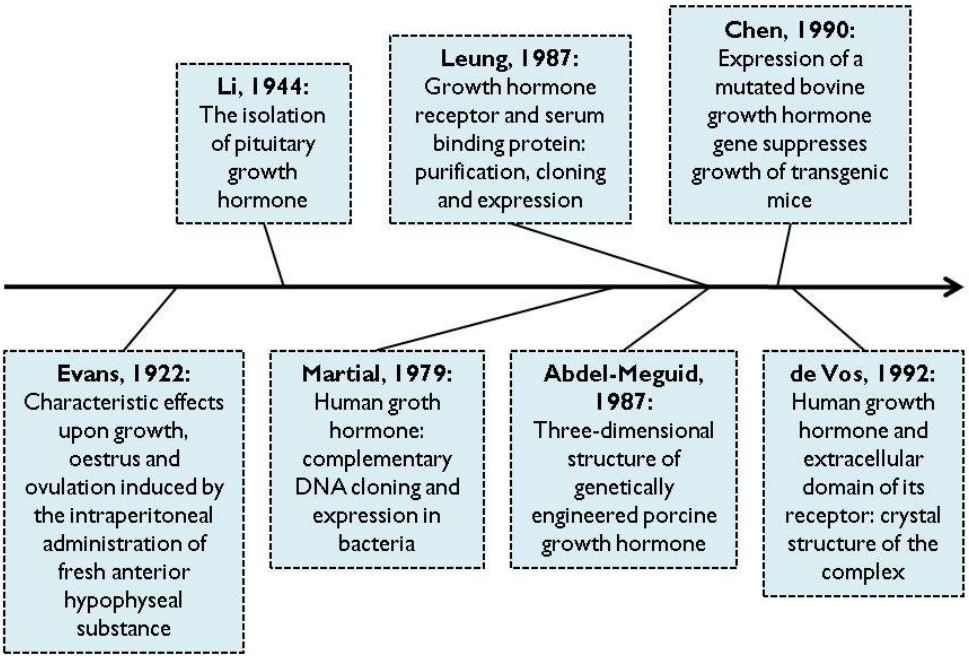

Figure 1: *Growth hormone time line.* Here are summarized the most important steps in growth hormone research, starting from discovery of a hypophyseal growth promoting substance in 1922.

In 1987, growth hormone receptor was purified and recombinantly expressed, and growth hormone binding protein was identified as its extracellular domain [Leung, 1987]. In the same year growth hormone was

for the first time crystallized [Abdel-Meguid, 1987]. In 1990 Chen et al. reported a growth hormone receptor antagonist, which nowadays is used as medication for acromegaly [Chen, 1990]. Crystal structure of the 1:2 complex formed by growth hormone and its binding protein was revealed in 1992 [de Vos, 1992].

1.1.2 Gene locus

The human growth hormone (hGH) gene cluster (see figure 2) on chromosome 17 (q22-24) contains the genes of normal pituitary-derived GH (*GH-N* or *GH-1*), its placental variant (*GH-V*) and three placental lactogens (PLs): products of *CS-A* and *CS-B* have the same amino acid sequence, *CS-L* encodes a shorter variant but a final product is not yielded. PLs are also known as chorionic somatomammotropins (CSs). *GH-1* is expressed mainly in the anterior pituitary whereas the others are expressed in placenta during gestation [Chen, 1989]. The differential expression is achieved via distinct locus control region (LCR) elements and epigenetic regulation [Ho, 2004].

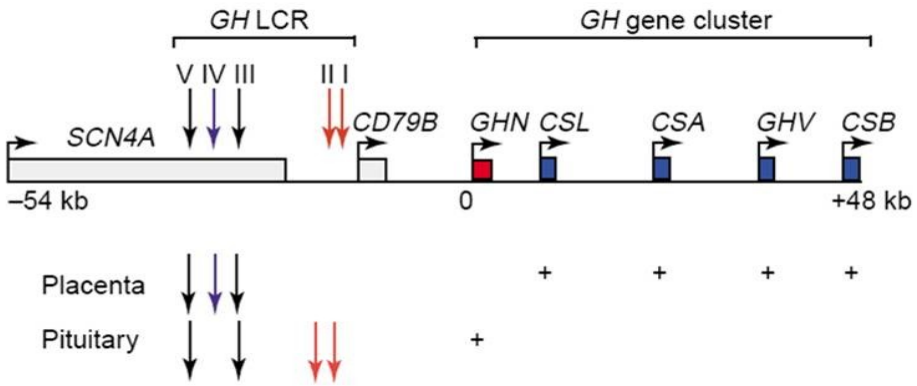

Figure 2: *The GH gene cluster.* Five genes are included in the GH gene cluster: *GH-N* (also known as *GH-1*) for pituitary GH, *GH-V* for placental GH and *CS-A*, *C-SB* and *CS-L* for placental lactogens. Blue colour refers to placenta and red to pituitary. There are five LCR elements regulating the expression of the GH gene cluster. Two of them (I and II) are pituitary-specific, one is placenta-specific (IV) and two operate in both tissues (III and V). In addition to the distinct LCR elements, also epigenetic regulation contributes to tissue-specific expression. *CD79B* and *SCN4A* are only included because the control region spans over them. *CD79B* encodes a sodium channel and *SCN4A* a B-cell receptor subunit. The figure was modified from Ho, 2004.

1.1.3 Structure

Mature hGH is a 22 kD protein consisting of 191 amino acids. There are several molecular forms of GH in addition to the predominating 22 kD GH (see chapter 1.1.4.1). The 191 amino acids are organised as an antiparallel four-helix bundle in an up-up-down-down manner, which is more rare than the simple up-down-up-down topology. Helices I (L6-Y35) and IV (A155-S184) are somewhat longer than helices II (N72-A98) and III (D107-R127) [Chantalat, 1995; de Vos, 1992; Sundström, 1996]. P89 causes a kink in helix II but this cannot be seen in the projection in figure 3.

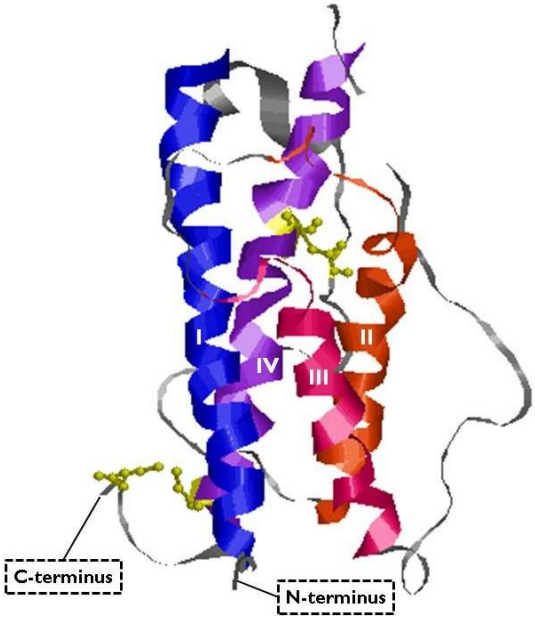

Figure 3: *A hGH molecule.* 22 kD hGH is a four-helix bundle with two disulfide bridges, which form the C-terminal small loop and the large loop between helix IV and the crossover connection between helices I and II. Even though it is not evident in this 3D model, the "top" of helix II starting from P89 is in reality also an α-helix, as indicated by the colouring. RasMol was used for molecular modelling. PDB ID of this crystal structure is 1HGU [Chantalat, 1995] but the helices are coloured based on two models of GH in complex with its binding protein (GHBP), namely 3HHR and 1HWG [de Vos, 1992; Sundström, 1996].

As can be expected from the up-up-down-down topology, the connecting loops between helices I & II and III & IV are much longer than the one between helices II & III. Even the connecting loops contain helical segments. The structure is stabilised by two disulfide bridges. The one between C53 and C165 links the so called large loop from the crossover connection be-

tween helices I and II to helix IV. The other disulfide bridge links the small loop between helix IV and C-terminus via C182 and C189 [Chantalat, 1995; de Vos, 1992; Sundström, 1996]. Abbreviations and structures of amino acids are listed in appendix I. Disulfide bridges will be covered in detail in chapter 1.2.

1.1.3.1 Relatives

GH belongs to a polypeptide hormone family, whose other members in humans are PLs and prolactin (PRL). GH family belongs to the large group of hematopoietic cytokines, which are all four-helix bundles with the special up-up-down-down topology, and bind in a similar manner to hematopoietic receptors. Other hematopoietic cytokines include many interleukins, erythropoietin and ciliary neurotrophic factor.

Unlike genes of the other GH family members, which are included in the GH gene cluster on chromosome 17, the *PRL* gene is located on chromosome 6 (p22.2-p21.3). Therefore it is not surprising that hPRL shares the lowest sequence identity with hGH, 24%. Placental hGH is 93% and hPL (the longer variant) 86% identical to pituitary GH. Human and bovine GH have 66% identity. Sequences and the conserved amino acids are presented in table 1. Sequences were obtained from SwissProt and analysed with FASTA sequence comparison tool. It is believed that GH, PLs and PRL have a common ancestor, from which PRL diverged already 400 million years ago, whereas GH and PLs diverged only 10 million years ago [Cooke, 1981; Goffin, 1996]. Cow and man on the other hand diverged approximately 75 million years ago [Forsyth, 2002].

There is some degree of cross-reactivity between the hormones of the GH family and those of different species. For instance, non-primate GH is not active in human but human GH is active in non-primates [Forsyth, 2007].

Primate GH can also bind and activate primate PRL receptor but not vice versa [Teilum, 2005].

Table 1: *Conservation of GH in relatives and between species.* Same amino acid is coloured yellow, a similar one turquoise. Cysteines forming the disulfide bridges of hGH are underlined. Sequences were obtained from SwissProt and aligned and compared with FASTA.

hGH	FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQ
hGH-V	FPTIPLSRLFDNAMLRARRLYQLAYDTYQEFEEAYILKEQKYSFLQNPQ
hPL	VQTVPLSRLFDHAMLQAHRAHQLAIDTYQEFEETYIPKDQKYSFLHDSQ
bGH	AFPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPEGQRYS-IQNTQ
hPRL	LPICPGGAARCQVTLRDLFDRAVVLSHYIHNLSSEMFSEFDKRYT---HGRGFITKAI
hGH	TSLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFL----RSVFANSLVY
hGH-V	TSLCFSESIPTPSNRVKTQQKSNLELLRISLLLIQSWLEPVQLL----RSVFANSLVY
hPL	TSFCFSDSIPTPSNMEETQQKSNLELLRISLLLIESWLEPVRFL----RSMFANNLVY
bGH	VAFCFSETIPAPTGKNEAQQKSDLELLRISLLLIQSWLGPLQFL----SRVFTNSLVF
hPRL	NS-CHTSSLATPEDKEQAQQMNQKDFLSLIVSILRSWNEPLYHLVTEVRGMQEAPEAI
hGH	GASDSNVYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDA--LLKN
hGH-V	GASDSNVYRHLKDLEEGIQTLMWRLEDGSPRTGQIFNQSYSKFDTKSHNDDA--LLKN
hPL	DTSDSDDYHLLKDLEEGIQTLMGRLEDGSRRTGQILKQTYSKFDTNSHNHDA--LLKN
bGH	GTSDR-VYEKLKDLEEGILALMRELEDGTPRAGQILKQTYDKFDTNMRSDDA--LLKN
hPRL	LSKAVEIEEQTKRLLEGMELIVSQVHPET-KENEIYP-VWSGLPSLQMADEESRLSAY
hGH	YGLLYCFRKDMDKVETFLRIVQCRSV-EGS-CGF
hGH-V	YGLLYCFRKDMDKVETFLRIVQCRSV-EGS-CGF
hPL	YGLLYCFRKDMDKVETFLRMVQCRSV-EGS-CGF
bGH	YGLLSCFRKDLHKTETYLRVMKCRRFGEAS-CAF
hPRL	YNLLHCLRRDSHKIDNYLKLLKCRII-HNNNC

In the following chapters, unless otherwise stated, GH refers to human pituitary growth hormone.

1.1.4 Biosynthesis

Practically all GH in circulation is synthesized by somatotrophs in anterior pituitary, except for during gestation when the placental GH variant predominates. The synthesis is controlled by several different stimuli, most importantly by the hypothalamic hormones GH releasing hormone

(GHRH) and somatostatin (SST), both signalling via G-protein coupled receptors (GPCRs). Control of GH secretion is subject of chapter 1.1.5. For long it was thought that somatotrophs are the only site of GH synthesis but according to current evidence GH is produced in several tissues, where it acts in an autocrine and paracrine manner [Gahete, 2009; Harvey, 1997]. Pituitary-specific transcription factor-1 (Pit-1) plays an important role in GH synthesis and in somatotroph differentiation. Its expression was also thought to be restricted to pituitary but, similarly as in case of GH, extrapituitary sites of expression, such as the mammary gland, are being identified [Gil-Puig, 2005].

The *GH-1* gene is preceded by a 26 amino acid signal sequence, which is typical for a secreted hormone [Strobl, 1994]. Translation of the mRNA transcript takes place on the rough endoplasmic reticulum (RER) whereby the preprotein is translocated into the RER lumen. The signal sequence is removed by a signal peptidase, the protein is folded and disulfide bridges are formed. The mature proteins migrate further to the Golgi apparatus, where they are packaged into secretory vesicles [Strobl, 1994; Voet, 2004]. The vesicles are released upon increased intracellular cyclic AMP (cAMP) and/or free Ca^{2+}. GHRH receptor is coupled to a $G\alpha_s$ and SST receptor to a $G\alpha_i$ subunit, ligand binding leading to activation or inhibition of adenylate cyclase, respectively [Strobl, 1994].

1.1.4.1 GH variants

In addition to the two genetic variants of GH, i.e. GH-N and GH-V, there are many other sources of variation. *GH-1* can go through alternative splicing and mature GH can be modified posttranslationally. The *GH-1* gene consists of five exons (E1-5), four introns (A-D) and untranslated regions (UTRs) in 5′ and 3′ termini, as seen in figure 4. Two splice variants, namely 22 and 20 kD GH, can be synthesised by alternative splicing of intron B/E3. After removal of the 26 amino acid signal sequence, 22 kD GH contains

191 and 20 kD variant 176 amino acids. Amino acids 32-46 lacking in 20 kD GH are located at the end of helix I and in the connecting loop between helices I and II. 20 kD GH comprises 5-10% of the GH produced in pituitary but ~15% of the GH in circulation because it has a higher stability than 22 kD GH [Baumann, 1999; Kopchick, 2007; Lewis, 1980 & 2000; Zhan, 2005].

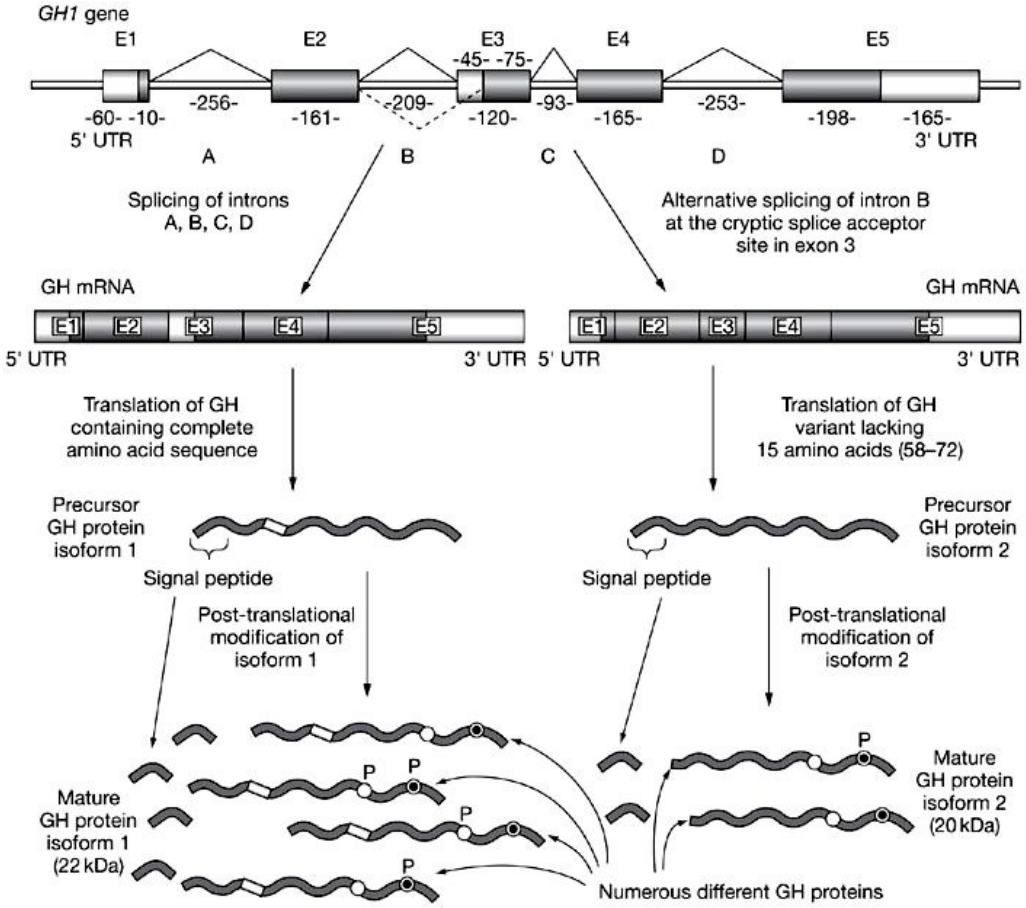

Figure 4: *Alternative splicing of GH-1*. All five exons (E1-5) of the *GH-1* gene are included in both splice variants, but their difference comes from alternative splicing within E3. UTR indicates an untranslated region. After removal of signal peptide, the 22 kD variant (isoform 1) contains 191 and the 20 kD variant (isoform 2) 176 amino acids. Several different posttranslational modifications are possible, phoshporylation (P) is presented as an example. The figure has been originally published by Kopchick [2007].

Splice variants must not be confused with circulating GH fragments that are result of GH proteolysis. These fragments are to some extent immunoreactive but their physiological roles are still under investigation. Some

fragments appear to have insulin-like but no growth-promoting activity [Baumann, 1999; De Palo, 2006(x2); Lewis, 2000].

1.1.4.1.1 Posttranslationally modified variants

Mature GH can undergo a number of posttranslational modifications. Placental GH is subject to *N*-linked glycosylation [Frankenne, 1990; Baumann, 1999] whereas pituitary GH can be *O*-glycosylated [Haro, 1996; Bustamante, 2009]. These glycosylations result in 25 and 24 kD proteins, respectively. Smaller modifications include phosphorylation, deamidation and acylation [Baumann, 1999].

1.1.4.1.2 Oligomeric variants

GH interacts with itself to form dimers and larger oligomers, for the most part non-covalently. Also covalent dimers and oligomers occur, most of them formed by interchain disulfide bonding [Baumann, 1999; Lewis, 1977]. GH homodimers are biologically active but to a lower extent than monomeric GH [Yang, 2008].

1.1.4.1.3 GH-GHBP complex

GH forms a complex with the GH binding protein (GHBP), which equals the extracellular domain of GH receptor (GHR). Approximately 50% of 22 kD GH in circulation is bound to GHBP, predominantly as a 1:1 complex. 20 kD GH has a somewhat lower binding affinity to GHBP but still 25-30% of it is GHBP-bound. GHBP protects GH from degradation and physiological clearance but at the same time keeps it unavailable for GHR [Baumann, 1988 & 1994].

1.1.5 From hypothalamus to periphery and vice versa

Hypothalamus is the master organ that plays the most essential role in controlling GH secretion. GHRH stimulates and SST inhibits GH release. There are however numerous other factors that are involved in the control of GH secretion. As summarized in figure 5, the other major regulators are ghrelin, leptin and free fatty acids (FFAs), GH itself and its second messenger insulin-like growth factor-1 (IGF-1). Additional hormonal and metabolic regulators include e.g. sex steroids, glucocorticoids and amino acids, which are not discussed here in further detail [Chowen, 2004; Müller, 1999]. GH secretion can be controlled either by influencing GHRH and SST release from hypothalamus, by interacting directly with the anterior pituitary, or by both mechanisms.

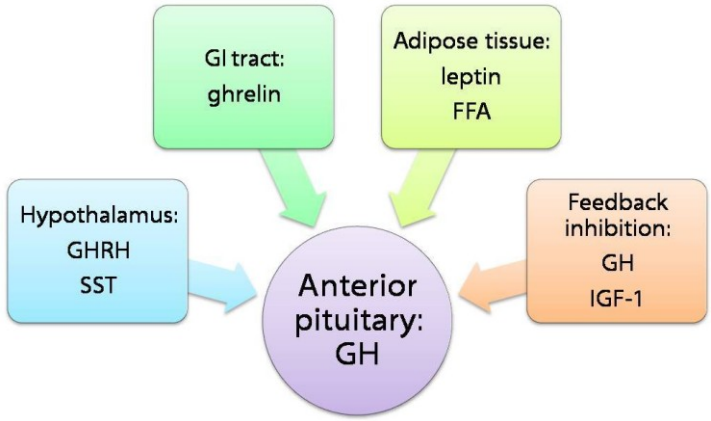

Figure 5: *Control of GH secretion.* GH release from anterior pituitary depends on a large amount of signals. Some of the signals act through hypothalamus.

1.1.5.1 Hypothalamic control of GH secretion

The hypothalamic hormones GHRH and SST regulate both *GH-1* gene transcription and GH release from the somatotrophs. Transcription control is mediated by various transcription factors, particularly Pit-1 [Müller, 1999; Pombo, 2001]. GHRH is mainly expressed in the arcuate nucleus and SST in the periventricular nucleus of hypothalamus [Fodor, 2006, Lopéz, 2010]. Their secretion is regulated hormonally and metabolically, most im-

portantly by GH feedback [Müller, 1999]. Hypothalamic interactions play an important role as well: orexinergic neurons mediate information about sleep-wake cycle and energy homeostasis [Lopéz, 2010].

1.1.5.2 Ghrelin

Ghrelin is a gastric peptide best known for its GH-releasing and orexigenic effects even though it has numerous other actions both on hypothalamus-pituitary level and in periphery. Ghrelin is secreted upon energy restriction and needs to be acetylated in order to bind to the growth hormone secretagogue receptor type 1a (GHS-R1a) [Ghigo, 2005; van der Lely, 2004]. GHS-R1a is very prominent in hypothalamus and pituitary and it is believed that ghrelin has its GH-releasing effect both by regulating hypothalamic GHRH and SST and by directly stimulating GH secretion from the pituitary. At the pituitary level ghrelin acts as a SST antagonist and also has a direct impact on GH-release but still its action on the hypothalamic level is believed to have a greater significance [Kineman, 2007; Maghnie, 2007; Tannenbaum, 2003; van der Lely, 2004]. The detailed interaction between ghrelin, GHRH and SST remains to be solved.

1.1.5.3 Adipose tissue

In opposite to ghrelin, leptin is a hormone that is secreted upon satiety. It is secreted from white adipose tissue as a response to sufficient energy stores and, as a result, circulating leptin levels correlate with body fat mass. Leptin signals via its receptor isoform b, ObRb, which is very prominent in hypothalamus, the main site of leptin action [Boguzewski, 2010; Kelesidis, 2010]. Leptin has been shown to be able to modulate GH secretion but its role is still somewhat unclear: fasting and thereby decreased leptin levels lead to increased GH levels in humans but decreased GH levels in rats. Obesity on the other hand results in impaired growth

hormone axis, whereby it is difficult to distinguish the effect of leptin [Gahete, 2009; Müller, 1999; Pombo, 2001].

Free fatty acids that are released from the adipose tissue after lipolysis inhibit GH secretion. They have been suggested to act directly on pituitary, inhibiting the action of GHRH [Alvarez, 1991; Pombo, 2001].

1.1.5.4 Feedback inhibition

GHRH, SST, GH itself and its ubiquitous second messenger IGF-1 form a complex feedback regulation of GH production, including long, short and ultrashort feedback loops [Gahete, 2009; Giustina, 1998; Müller, 1999]. GH regulates its own production in an autocrine/paracrine manner at the pituitary level but also via a long feedback loop [Gahete, 2009]. Mechanism of the autofeedback action of GH is not completely clear but GH is known to stimulate SST and inhibit GHRH, thus inhibiting its own production [Asa, 2000; Giustina, 1998; Müller, 1999]. IGF-1 acts along GH in the long feedback loop by stimulating SST release from hypothalamus and directly inhibiting GH release on pituitary level [Gahete, 2009; Müller 1999]. Both GH and IGF-1 can to some extent cross the blood-brain barrier, enabling their direct interaction with the hypothalamus [Pan, 2005; Reinhardt, 1994].

1.1.5.5 GH action

A simplified version of GH action is presented in figure 6. Once GH enters the circulation it is transported to target organs, partly bound to GHBP [Baumann, 1990]. GHR mRNA is expressed in various human tissues, above all in liver, adipose tissue, muscle and kidney, indicating high GH activity in these tissues [Ballesteros, 2000; Kaplan, 2007]. GH is best known for its growth promoting effects but it has also a number of metabolic actions.

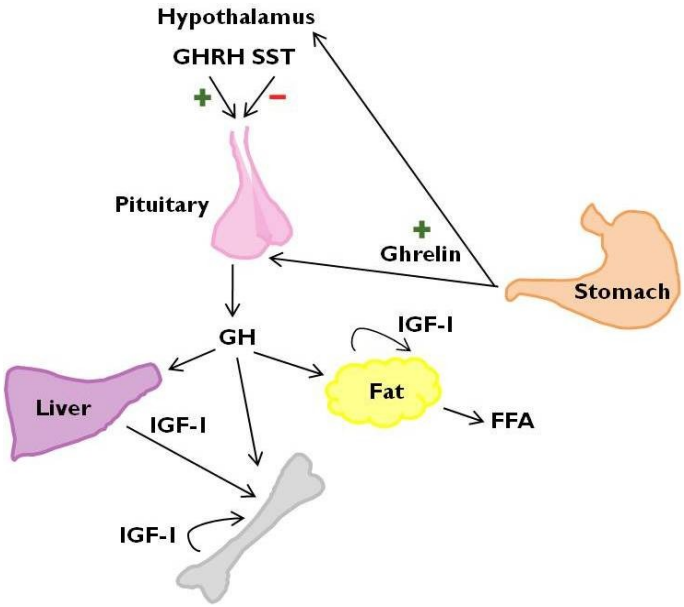

Figure 6: *GH-axis.* GH release from anterior pituitary is regulated among others by GHRH, SST and ghrelin. In circulation approximately 50% of GH is bound to GHBP. In the target organs GH has metabolic actions and growth-promoting effects, which are mediated by IGF-1. Liver is an important site of IGF-1 production but it is also produced locally at target tissues.

1.1.5.5.1 Growth

GH markedly promotes skeletal muscle growth and longitudinal bone growth and remodelling [LeRoith, 2001]. These effects are mediated by IGF-1, also known as somatomedin. According to the original *somatomedin hypothesis*, GH-induced IGF-1 secretion from the liver was responsible for the growth-promoting effects of GH. In the updated hypothesis an important role is given to local IGF-1 production upon GH stimulation and its autocrine/paracrine action. In addition GH stimulates synthesis of IGF binding protein-3 (IGFBP-3) and acid-labile subunit (ALS), which are important IGF-1 carriers in circulation [Kaplan, 2007; LeRoith, 2001; Rosenfeld, 2009; Yakar, 1999].

1.1.5.5.2 Metabolism

The various metabolic actions of GH are summarized in table 2. Some but not all of them are mediated by IGF-1 but for most part the effects are inseparable [Mauras, 2005]. It is undisputed however that GH has a direct

anti-insulin effect on carbohydrate metabolism whereas IGF-1 has an insulin-like effect [LeRoith, 2007; Mauras, 2005]. GH reduces glucose uptake, stimulates gluconeogenesis and increases mobilization of lipids by stimulating lipolysis and β-oxidation and inhibiting lipogenesis. The role GH has in protein metabolism is metabolically less significant – the promotion of protein synthesis in muscle can be considered as a growth promoting effect [LeRoith, 2001 & 2007; Mauras, 2005; Møller, 2009]

Table 2: *Metabolic actions of GH.* It is not specified which effects are IGF-1-mediated. However, the effect on glucose metabolism is by GH alone [LeRoith, 2001 & 2007; Møller, 2009].

	+	-
Carbohydrates	gluconeogenesis	glucose uptake
Proteins	protein synthesis	
Lipids	lipolysis β-oxidation ketogenesis	lipogenesis

1.1.5.5.3 Immunology

Lymphocytes are both a target and a production site of GH. GH stimulates thymopoiesis, proliferation of T and B cells and production of cytokines. Furthermore it increases macrophage and neutrophil activity and inhibits apoptosis [Hattori, 2009; LeRoith, 2001]. The immunomodulatory effects of GH are at least to some extent mediated by IGF-1 [Dorshkind, 2000; Hattori, 2009].

1.1.5.5.4 Renal function

GH, via IGF-1, reduces renal vascular resistance and increases renal plasma flow and glomerular filtration rate, leading to hyperfiltration. GH

also increases renal sodium and fluid retention, which could lead to hypertension at elevated GH levels [Ogle, 1992(x2)].

1.1.6 GH signalling

1.1.6.1 Growth hormone receptor

Most actions of GH are launched by GH binding to the GH receptor, GHR. Full-length GHR has 620 amino acids. N-terminal residues 1-246 form the extracellular domain (ECD), which equals GHBP. C-terminal residues 271-620 form the intracellular domain (ICD), and the amino acid portion in between consists of hydrophobic amino acids which reside in the transmembrane region [Godowski, 1989; Leung, 1987]. 3D structure of the ICD is not known whereas the ECD is made up of two fibronectin type III domains, as can be seen in figure 7 [de Vos, 1992; Brooks, 2007]. Human GHBP is generated by proteolytic cleavage from the GHR but in rodents it is a splice variant of GHR [Baumann, 1994].

There are three known GHR isoforms besides the full length GHR: GHR1-277, GHR1-279 and GHR-d3, in which exon 3 (out of 1-10) is skipped, resulting in loss of amino acids 7-38. Biological properties of GHR-d3 do not differ from the full-length GHR [Seidel, 2003]. Since GHR1-277 and GHR1-279 lack most of the ICD, they cannot initiate signal transduction. GHR1-279 is more common (<10% of total GHR in liver, fat and muscle) than GHR1-277 (<1%) but both seem to have a very similar biological significance: they are dominant-negative inhibitors of the full-length receptor and shed large amounts of GHBP [Ballesteros, 2000; Ilda, 1999; Ross, 1997].

GHR is a class I cytokine receptor, and thus has no intrinsic kinase activity and requires associated kinases for tyrosine phosphorylation [Wells, 1996]. Tyrosine phosphorylation is the first step in signal transduction, which is the subject of chapter 1.1.6.2. For a long time it was believed that ligand binding causes the receptor to dimerize but it turned out that the receptor

is present on cell surface as a constitutive dimer [Gent, 2002] and ligand binding induces a conformational change which triggers intracellular kinase activation [Brooks, 2007; Brown, 2005]. Upon GH binding a disulfide bond is formed between C241 residues of the GHR ECDs but this linkage is not necessary for initiation of the intracellular signal transduction [Brown, 2005; Zhang, 1999]. GH binding to a pre-existing GHR dimer and the consequential conformational change are depicted in figure 7.

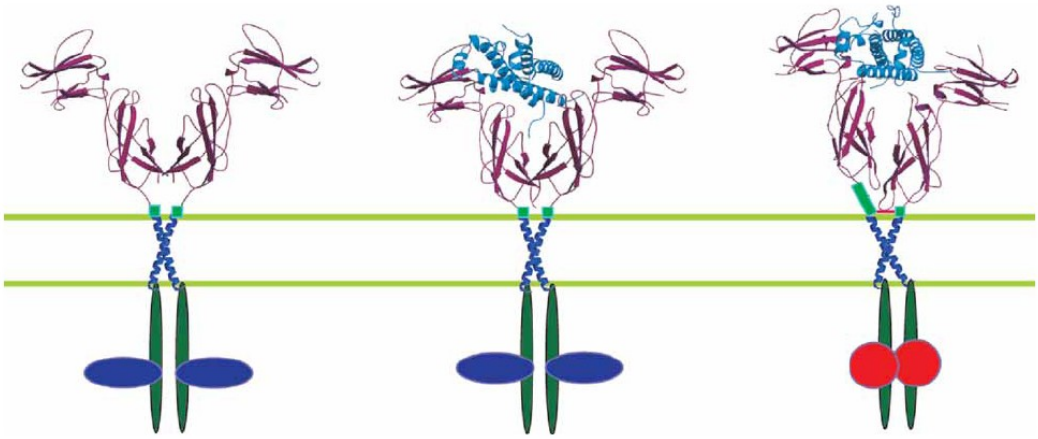

Figure 7: *GHR activation.* GHR is found on the cell surface as a pre-existing dimer. GH binding results in a conformational change that triggers intracellular signalling. The blue ellipses are inactivated Jak2s (Janus kinase 2, see chapter 1.1.6.2), which become activated (red spheres) upon ligand binding. The red line represents a disulfide bond, which is formed at receptor C241 as a result of the conformational change [Brown, 2005].

1.1.6.1.1 GH binding site 1 and 2

GH has two GHR binding sites, which bind to roughly the same site on the receptor [Cunningham, 1991; Wells, 1996]. The relevant amino acids for each binding site on GH as determined by Cunningham are indicated on figure 8. Binding site 1 has very high affinity to GHR whereas the affinity of binding site 2 is much lower. In alanine mutation studies 22 amino acids were reported to be critical for site 1 binding but only five for site 2 binding. Helix II does not contain such amino acids, helix I contains amino acids that are essential for both binding sites, helix III is important for binding site 2 and helix IV for binding site 1 [Cunningham, 1991].

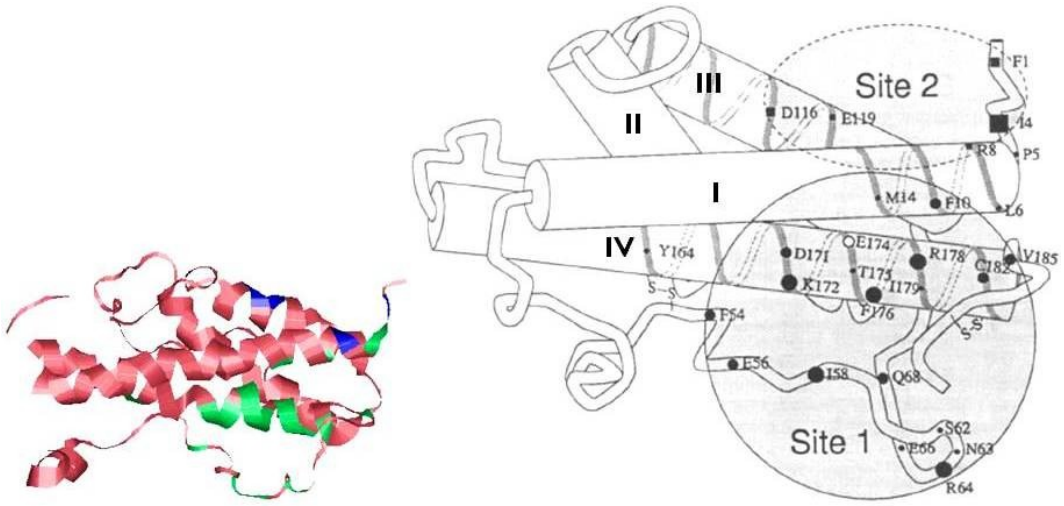

Figure 8: *GHR binding sites of GH.* GHR binding site 1 is sea green and 2 blue on the pink GH molecule, which is here in the same position as the model by Cunningham [1991] containing the amino acids that are important for binding at each site. Helix II doesn't include critical amino acids, helix I contains amino acids that are essential for both binding sites, helix III is important for binding site 2 and helix IV for binding site 1. Molecular modelling of PDB entry 1HGU [Chantalat, 1995] was done with RasMol.

GH binds to GHR sequentially: site 2 binding does not occur until binding site 1 is associated with receptor [Cunningham, 1991; de Vos, 1992]. This is due to an allosteric change caused by site 1 binding [Walsh, 2004].

1.1.6.2 Signal transduction

Janus kinases (Jaks) are a family of receptor kinases that mediate signalling of cytokine receptors. The family consists of Jak1, 2 and 3 and tyrosine kinase 2 (Tyk2), of which Jak2 interacts with GHR [Rosenfeld, 2009; Wells, 1996]. Each GHR ICD can be associated with one Jak2, which is activated upon ligand binding. The conformational change brings the two ICDs and thereby the Jak2s closer together, which allows the Jak2 on one GHR ICD to transphosphorylate the Jak2 on the other. Consequently several tyrosine residues on the GHR are phosphorylated, launching signal transduction pathways [Brown, 2005; Lanning, 2006; Waters, 2006].

1.1.6.2.1 Jak/STAT pathway

The so called Jak/STAT pathway (see figure 9) is the major signal transduction pathway in GH signalling. The role of Jak (Jak2) was already presented above. STATs are a family of "signal transducers and activators of transcription", consisting of STATs 1, 2, 3, 4, 5a, 5b and 6. STAT1 and STAT3 can to some extent be activated by GHR but STAT5a and STAT5b are the most critical STATs for GH signalling [Paukku, 2004; Rosenfeld, 2009; Wells, 1996].

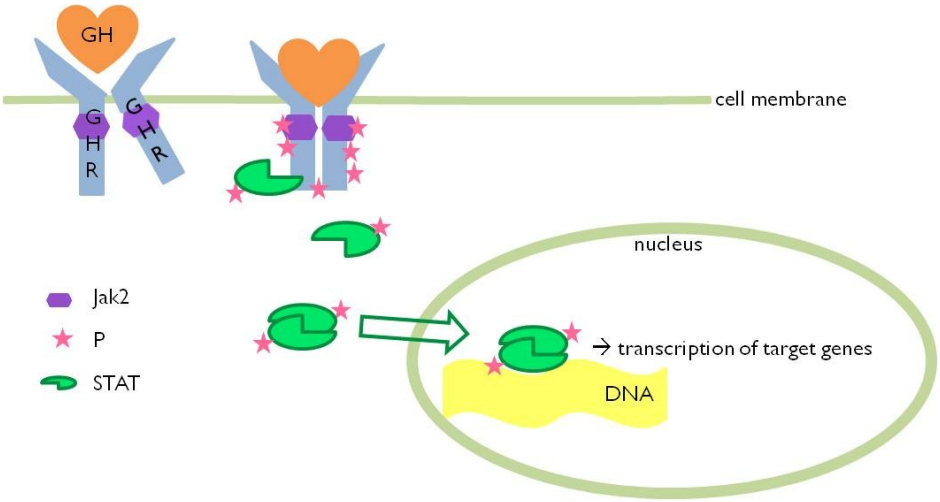

Figure 9: *Jak/STAT signalling pathway.* Jak2 bound to GHR is activated upon ligand binding, which leads to phosphorylation of Jak2 on the adjacent GHR. Consequently also the GHR and STAT bound to it are phosphorylated. Phosphorylated STAT5 homo/heterodimerizes and translocates into the nucleus, where it activates the transcription of target genes.

STATs bind to the activated GHR-Jak2 complex, where they as well are phosphorylated and thereby activated. Activated STATs form homo- or heterodimers, which are translocated into the nucleus, where they function as transcription factors [Lanning, 2006; Rosenfeld, 2009]. The activated genes include e.g. *IGF-1* [Cesena, 2007; Woelfle, 2003] and certain members of a family of suppressors of cytokine signalling (SOCS). SOCS can either bind to Jak2 or GHR, hampering the activating phosphorylations, or block STAT5 binding sites on GHR [Croker, 2008; Lanning, 2006].

1.1.6.2.2 Additional pathways

Activation of Jak2 can initiate at least two other pathways in addition to the Jak/STAT pathway, namely the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Signalling may also take place Jak2-independently via Src tyrosine kinases which also leads to activation of the MAPK pathway. Also these pathways lead to activation of target genes [Brooks, 2007; Lanning, 2006]. However, these additional pathways are not enough to maintain the necessary GH-induced IGF-1 levels in absence of STAT5 [Rosenfeld, 2009].

1.1.6.3 Recycling of GHR

Half-life of GHR is approximately 1 h [Brooks, 2007]. GHR is recycled by ubiquitin-dependent internalization and lysosomal degradation of the GHR-GH-GHR complex [Frank, 2008; van Kerkhof, 2007]. GHR1-279 is internalized at a much lower rate than full-length GHR, which accounts for its dominant negative action [Maamra, 1999]. Another means for recycling is the proteolytic shedding of GHBP followed by release of the ICD into the cytosol [Frank, 2008]. The ICD remnant has been suggested to have GH-independent roles e.g. in regulation of gene transcription [Cowan, 2005].

1.1.7 GH in health and disease

1.1.7.1 Normal GH levels

GH is secreted in a pulsatile manner and is stimulated above all by sleep and exercise [Müller, 1999; Strobl, 1994]. GH levels are highest during puberty [Strobl, 1994] and then during adult life decrease 14% per decade [Toogood, 1996]. Changes in GH secretion do not affect the intervals between pulses but rather their amplitude [Müller, 1999; Strobl, 1994]. GH is secreted on average 12 times a day and since its half-life is only 15-18 min-

utes, the basal GH levels are very low. Mean (24h average) serum GH concentration is 0.8 µg/l in women and 0.3 µg/l in men and peak values are 2.1 µg/l and 0.7 µg/l, respectively [van den Berg, 1996].

1.1.7.1.1 The sex factor

GH levels are 2-3-fold higher in women than in men. Women have a higher maximal GH plasma concentration and their GH pulses are prolonged while the frequency remains the same as in men [van den Berg, 1996]. Also exercise-induced GH secretion is more notable in women than in men [Strobl, 1994]. These differences are at least partly mediated by oestrogen [Chowen, 2004; Leung, 2004; Strobl, 1994]. 24-h curves of female vs. male GH plasma concentrations are shown in figure 10. Secretion rates were calculated based on the plasma concentrations by "multiparameter deconvolution analysis" [van den Berg, 1996].

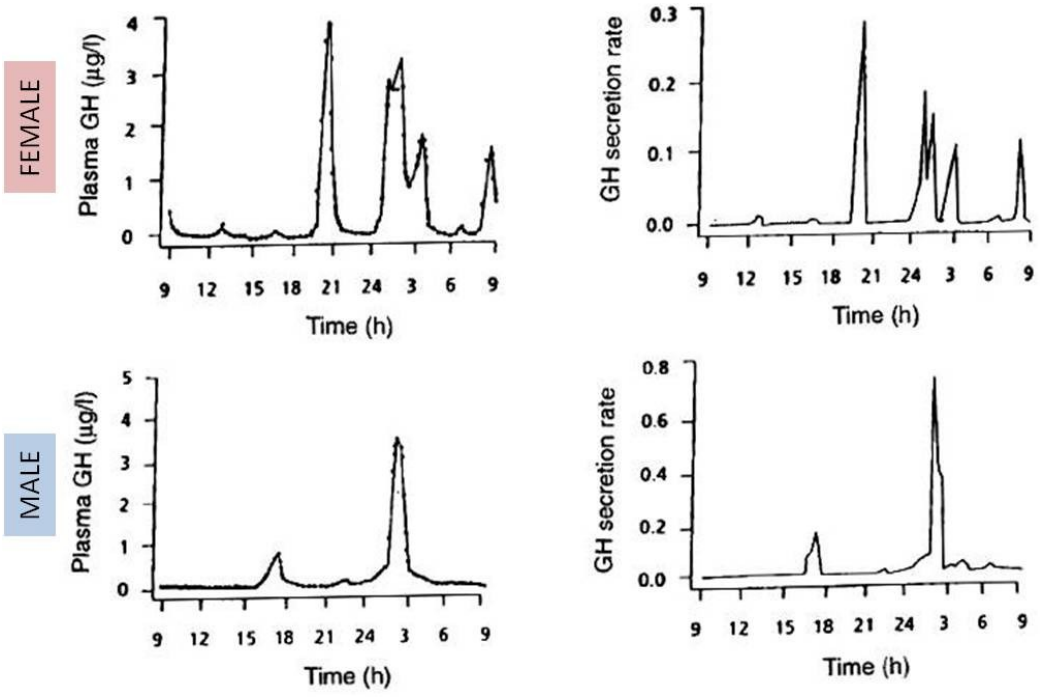

Figure 10: *24-h plasma GH levels.* Samples were collected at 10-min intervals and their concentrations were measured by a highly sensitive immunofluorometric assay. GH secretion rate was calculated from plasma GH concentration by multiparameter deconvolution analysis [van den Berg, 1996].

1.1.7.1.2 Gestation

Pituitary GH concentration is reported to decrease during gestation, being partially replaced by placental GH. Overall GH level is rather low during the transition period, contributing to storage of fat [Freemark, 2006].

GH seems to be rather unimportant for prenatal growth – unlike IGF-1. This can be observed in birth weights of neonates with either GH/GHR or IGF-1/IGF-1 receptor deficiencies: only the latter have retarded intrauterine growth whereas postnatal growth will be impaired in both cases [Rosenfeld, 2009].

1.1.7.2 Excess GH

Acromegaly is a rare condition, where elevated GH levels cause overgrowth of extremities and soft tissue hypertrophy. Typical facial features of acromegalic patients can be seen in figure 11. Symptoms and consequences include e.g. sweating, joint pain, sleep apnoea, hypertension and diabetes. Basal (between peaks) plasma GH concentration in acromegalic patients is above 0.4 μg/l (see figure 10) [Chanson, 2008]. Childhood GH excess is called gigantism: as long as the epiphyseal growth plates are open, high GH levels result in excessive linear growth [Eugster, 1999]. Somatotroph pituitary adenomas are the most common cause in both acromegaly and gigantism. Extrapituitary causes include impaired GHRH or SST secretion [Chanson, 2008; Eugster, 1999]. These conditions are primarily treated by surgery but if it cannot be accomplished or does not normalise GH levels, radiotherapy and/or medical treatment is applied. The latter includes SST analogues and dopamine agonists, dopamine being one of the hypothalamic mediators of SST release [Bush, 2008; Chanson, 2008; Müller, 1999]. A rather new approach is the use of a GHR antagonist, Pegvisomant.

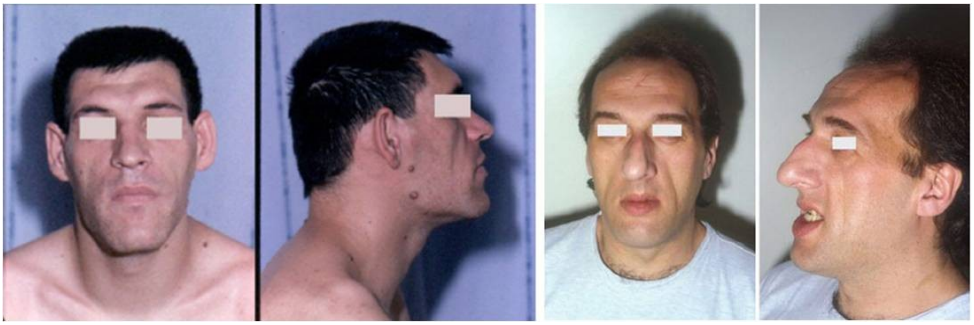

Figure 11: *Typical features of acromegalic patients.* Bulge in the forehead, big nose, prominent jaw and thick lips and tongue are common outer signs of excess GH [Chanson, 2008].

1.1.7.2.1 Pegvisomant

Pegvisomant is an analogue of human GH but the mutation G120K makes it a GHR antagonist. The antagonist effect of such a mutation was discovered in 1990 [Chen, 1990]. G120 is located in proximity of GH binding site 2 and replacing the tiny glycine with a bulkier amino acid prevents correct GHR binding on this binding site. The original findings were based on a G120R mutation but the mutation currently in use is G120K. Since the G120K mutant cannot initiate the intracellular conformational change required for signal transduction but still occupies the receptor via high-affinity binding on binding site 1, it acts as a GHR antagonist. The antagonistic effect and medical applicability of the molecule have been improved by adding eight additional mutations that enhance site 1 binding and 4-6 polyethylene glycol (PEG) residues to increase the half-life [Kopchick, 2002]. On the other hand, the PEG residues cause a 30-40-fold reduction in binding affinity to GHR but this does not outweigh the benefits [Pradhananga, 2002; Ross, 2001]. Pegvisomant treatment of acromegaly has proven very efficient: 90% of subjects reach normal IGF-1 levels. The high dose up to 40 mg/day was an initial concern but no severe side effects have been reported so far [Higham, 2008; Kopchick, 2002].

1.1.7.2.2 GH abuse in sport

Because of its protein anabolic effects, GH is of interest for athletes and bodybuilders. Recombinant human 22 kD GH (rhGH) is being used as a doping agent even though its efficacy has not been proven and it may lead to a condition similar to acromegaly [Saugy, 2006]. Detection of GH abuse is complicated because rhGH does not differ from the endogenous hormone and its half-life is rather short. However, upon exogenous GH administration, the normal mixture of GH variants is impaired and the usage of rhGH can be detected using high-sensitivity immunoassays that measure the proportions of GH isoforms, especially 20 vs. 22 kD GH [Bidlingmaier, 2009].

1.1.7.3 GH deficiency and Kowarski syndrome

There are four types of familial isolated GH deficiency (IGHD), caused by a variety of genetic disorders resulting in impaired GH secretion. The classes of IGHD, their inheritance and cause are summarized in table 3. Diagnosis of childhood IGHD is complex but a height more than 3 SD below mean and a peak GH value <10 μg/l after provocation test are typical criteria [GH Research Society, 2000].

Table 3: *Classification of GH deficiency.*

IGHD type	Inheritance	[GH]	Candidate gene
IA	recessive	absent	*GH-1*
IB	recessive	low	*GH-1*, *GHRH*, *GHRHR* etc.
II	dominant	low	*GH-1*
III	X-linked	low	unknown

In IGHD type IA the *GH-1* gene is deleted or silent due to frameshift or nonsense mutations. This condition is recessively inherited and leads to severe dwarfism by the age of six months [Mullis, 2007]. As can be con-

cluded by the recessive inheritance, one functional *GH-1* gene suffices for normal growth [Binder, 2002]. Since GH is completely absent in IGHD type IA patients, treatment with rhGH is problematic due to generation of anti-GH antibodies [Mullis, 2007]. IGHD type IB is also recessively inherited but less severe than type IA due to detectable GH levels. In addition to *GH-1*, also genes encoding GHRH, its receptor (GHRHR) or regulators of *GH-1* gene transcription may be altered [Mullis, 2007].

IGHD type II differs from type I particularly by its dominant inheritance. Most common cause is any of several mutations of the *GH-1* gene, which cause skipping of exon 3 (Δexon3), i.e. amino acids 37-71, and result in a 17.5 kD GH. This variant has a dominant negative effect by interfering with 22 kD GH secretion [Mullis, 2007]. Also missense mutations of *GH-1* have been reported to cause IGHD type II, for instance P89L, V110L and R183H, which all substitute extremely conserved amino acids (see table 1) [Binder, 2001; Mullis, 2007; Salemi, 2005]. Their positions on a GHBP-GH-GHBP complex can be seen in figure 12.

IGHD type III is the rarest kind of IGHD and has an X-linked recessive inheritance. It is caused by alterations in the X chromosome, which probably are related to genes involved in immunoglobulin production since these patients are also missing immunoglobulins [Mullis, 2007].

Kowarski syndrome is caused by bio-inactive GH. It differs from IGHD by normal to high GH levels and from Laron syndrome by functional GHR [Kowarski, 1978]. In Laron syndrome growth retardation is due to defects on GHR level [Laron, 1966]. Three *GH-1* mutations have been reported to cause Kowarski syndrome, a homozygous C53S mutation [Besson, 2005] and heterozygous R77C and D112G mutations [Chihara, 1998; Takahashi, 1996 & 1997]. Heterozygous I179M [Lewis, 2004] and D116E [Dateki, 2009] mutations have also been identified in patients with short stature but no

evidence for bio-inactivity was found. Positions of these missense mutations on a GHBP-GH-GHBP complex are indicated in figure 12.

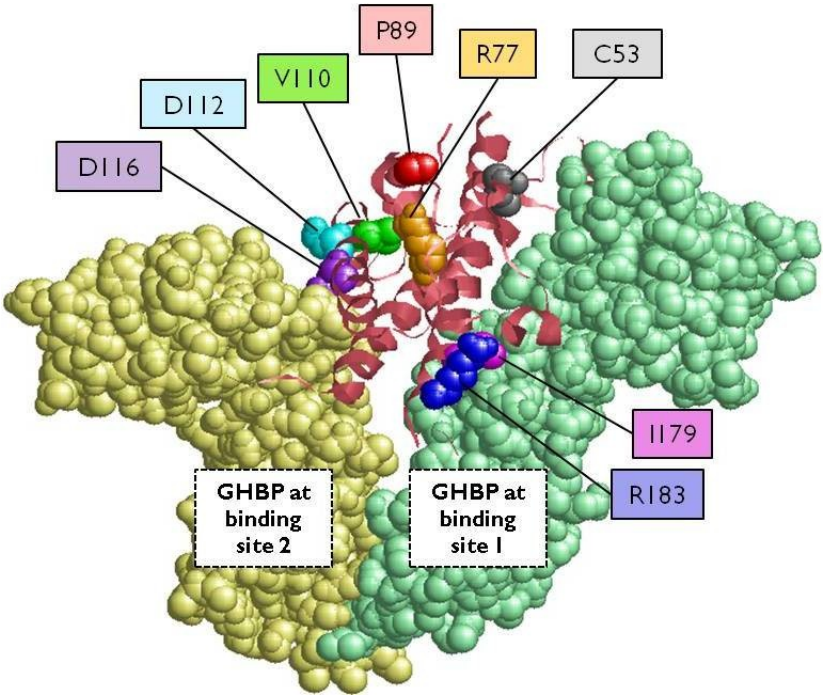

Figure 12: GH-1 *missense mutations associated with short stature*. A pink GH molecule is bound to two GHBPs, light yellow on binding site 2 and light sea green on binding site 1. Amino acids which undergo substitution mutations in patients with short stature are indicated. The corresponding mutations are C53S, R77C, P89L, V110L, D112G, D116E, I179M and R183H. RasMol was used for molecular modelling. PDB ID of the 3D model is 1HWG [Sundström, 1996].

1.1.7.3.1 A novel *GH-1* deletion mutation leading to short stature

Some years ago a novel *GH-1* mutation was identified in twin girls, who were born in 1990, and in their mother and her father. They were first diagnosed with GH deficiency based on heights between -3.76 and -2.69 SD and peak GH concentrations between 6.6 and 7.3 µg/l [Weigel, 2004]. Their *GH-1* was sequenced and a heterozygous deletion of nine base pairs was revealed, resulting in deletion of three amino acids at C-terminus, Δ188-190. Unfortunately the girls did not respond very well to rhGH therapy but still final heights of 148 and 150 cm were achieved [unpublished data]. It was thought that the phenotype was similar to that of Δexon3 patients [Weigel, 2004] until it turned out that the GH concentrations had actually

been unusually high but could not have been detected in a conventional GH assay. Measurements done in our group using highly sensitive fluorescent immunoassays revealed GH levels up to 50 μg/l. The reason why the patients' GH could not have been detected in the conventional assay was that approximately 80% of it was in dimeric form, as shown by Western blot, fast protein liquid chromatography (FPLC) and immunoassays employing monoclonal antibodies that are able to distinguish between GH monomer and dimer.

Since many of the mutations associated with short stature involve the disulfide bridges of GH, a closer look is taken into the contribution of disulfide bridges for protein – and especially GH – structure in chapter 1.2.

1.2 Disulfide bridges

1.2.1 Formation and contribution to structure

The amino acid cysteine has the unique ability to form disulfide bonds to other cysteine residues through oxidation of their thiol groups:

$$\mathbf{R\text{-}SH + HS\text{-}R \rightarrow R\text{-}S\text{-}S\text{-}R + 2H^{+} + 2\,e^{-}}$$

The reaction occurs spontaneously in an oxidising environment but can also be assisted by protein disulfide isomerases (PDIs), which catalyse disulfide interchange reactions. Disulfide bridge formation during protein synthesis takes place in the oxidising environment of ER [Voet, 2004]. Disulfide bonds are not needed for correct folding of proteins but rather stabilize the correct 3D structure [Betz, 1993; Voet, 2004].

1.2.2 Disulfide bridges in GH

As already mentioned in chapter 1.1.3, the GH family members have a four helix bundle structure with an up-up-down-down motif. Pituitary

and placental hGH, hPL, hPRL and bGH, which were aligned in table 1, all have a disulfide bridge linking the crossover connection between helices I and II to helix IV. They all also have a C-terminal small loop and hPRL has an additional small loop at N-terminus [Chantalat, 1995; Teilum, 2005; Walsh, 2006]. The disulfide bridges are conserved not only throughout these family members but also more extensively throughout species [Watahiki, 1989; Nicoll, 1986].

The disulfide bridge between C53 and C165 has shown to be essential for both GH secretion and function [Besson, 2005; Chen, 1992; Gráf, 1976; Iliev, 2005]. The C-terminal disulfide bridge between C182 and C189 has been reported to be unimportant for biological potency, secretion and receptor binding but has not been under as much investigation as the other disulfide bridge [Chen, 1992; Gráf, 1976]. The conservation still speaks for its importance.

Several of the mutations that are associated with short stature somehow interfere with the disulfide bridges of GH. C165 is deleted as a consequence of Δexon3 mutations causing IGHD type II [Mullis, 2007] and its counterpart, C53, is mutated in the case of Kowarski syndrome caused by a homozygous C53S mutation [Besson, 2005]. R183H mutation that is also associated with IGHD type II is adjacent to the C182 of the C-terminal disulfide bridge and may interfere with its formation [Binder, 2001]. In addition, a C182R mutation has been identified in a hypertension patient but the mutant did not differ from wild type (wt) GH in *in vitro* experiments and causality could not be shown [Horan, 2006]. Finally, the C-terminal disulfide bridge is disrupted in the novel heterozygous mutation Δ188-190 due to deletion of C189. These mutations being able to cause short stature indicate an important role for the disulfide bridges – also for the one in C-terminus.

2 Aim

The primary aim of this PhD project was to characterize the novel GH deletion mutant Δ188-190 and to reveal its mechanism of causing short stature. The possible antagonistic effect of Δ188-190 on GHR was especially under focus. Firstly the mutant had to be produced *in vitro* and secondly suitable methods for its characterization had to be established. Important aspects for characterization of GH variants and mutants are expression and secretion, binding affinity to GHR and to GHBP, biological activity and stability. Since the novel mutation disrupted the C-terminal disulfide bridge of GH and since the role of this extremely conserved structural feature was rather unknown, we decided to take a closer look into its contribution to the molecule as well. This was done by substituting the involved cysteines with alanines. GH mutants that had been reported to be associated with short stature were another matter of interest. We chose to investigate R77C because it had been suggested to be a GHR antagonist although this hypothesis has meanwhile been proven wrong. We were curious about D112G since it had only been characterized as it was first reported in 1998 and was claimed to be biologically inactive. We therefore investigated the properties of the new mutant Δ188-190 in comparison to previously published GH mutants and newly designed GH mutants with a modified C-terminus.

3 Materials and methods

Suppliers of the chemicals and reagents, laboratory equipment, kits, consumables and software used in this project can be found in appendix III.

3.1 Recombinant DNA work

First practical step of this project was to construct expression plasmids containing either mutated or wt human *GH-1* gene. Compositions of working solutions needed for the recombinant DNA work are listed in table 4.

Table 4: *Compositions of the working solutions in chapter 3.1 (*was prepared sterile).*

Agarose gel, 1%	✓ 1% agarose (w/v) in 1x TBE, dissolved by boiling ✓ 1:10,000 CYBR® Safe DNA gel stain added once cooled down
TBE, 10x	✓ 0.89 M Trizma® base, 0.89 M boric acid and 20 mM EDTA in ddH_2O ✓ autoclaved
Loading dye, 6x	✓ 0.25% bromophenol blue (w/v) and 30% glycerol in TE buffer ✓ short-term storage at 4°C, long-term in -20°C
TE buffer	✓ 10 mM Trizma® base and 1 mM EDTA in ddH_2O ✓ pH 8.0 (HCl) ✓ autoclaved
Lysogeny broth/ampicillin (LB/amp) medium	✓ 1 LB Broth tablet per 50 ml ddH_2O ✓ autoclaved ✓ stored at 4°C ✓ 100 µg/ml ampicillin added before use
Ampicillin, 10 mg/ml*	✓ 20 mg ampicillin in 20 ml sterile ddH_2O ✓ filtrated with a 0.2 µm filter ✓ stored in -20°C
LB/amp plates*	✓ 1 LB Agar tablet per 50 ml ddH_2O ✓ autoclaved ✓ 100 µg/ml ampicillin added once cooled down to ca 55°C ✓ ca 20 ml poured per Petri dish

3.1.1 Site-directed mutagenesis

Plasmid pGH-Sec-Tag-GH-stop containing the human *GH-1* gene preceded by 26 aa signal sequence [Strobl, 1994] was a kind gift from Dr. Ian Wilkinson (Sheffield, UK). Sequence of *GH-1* and signal sequence can be found in appendix V. The *GH-1* gene was amplified and mutated by PCR. The GH forward primer included a 5′ CACC, which functions as a recognition sequence in directional TOPO cloning, which is described in chapter 3.2.2.

Mutations C182A, C189A and Δ188-190 were introduced by using a reverse primer containing the mutation in question. In case of R77C and D112G, the mutagenesis had to be performed in two steps. First the mutation was introduced in two overlapping segments using GH forward and mutated reverse primer for one segment (IA in figure 13), and mutated forward and GH reverse primer for the other (IB in figure 13). Then these two segments were used as template in a subsequent PCR employing GH forward and reverse primers (II in figure 13).

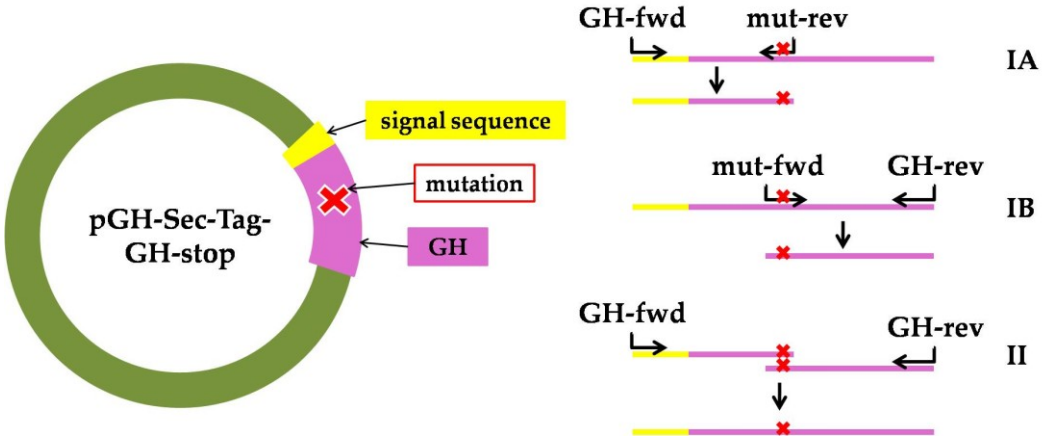

Figure 13: *Two-step PCR.* The DNA strands represent double-stranded template and PCR-product and not the actual steps of PCR. Fwd refers to forward and rev to reverse primer, and mut to mutant.

Mutation C182+9A was introduced by using the C182A mutated *GH-1* gene as template and the reverse primer containing the C189A mutation.

All primers are listed in appendix IV. Mutagenesis and cloning of the D112G mutant were performed by Friederike Braig during her Bachelor's thesis project. C182A and C189A were generated by Adna Halilovic and Heike Wobst during their Molecular Medicine Module II practical training. Ingredients of each PCR reaction and the used program are listed below. Template was either 10 ng plasmid pGH-Sec-Tag-GH-stop or 5 ng of both first round PCR products. Concentration of dNTP mix refers to the final concentration of each nucleotide. *Pfu* DNA polymerase, which has proof-reading ability, was used because blunt-ends were required for construction of the expression vector (see chapter 3.1.2). PCR was run on a Mastercycler Personal.

PCR composition	**Program**	
10 ng template	2' 94°C	
200 nM fwd & rev primers	30" 94°C	30x
200 µM dNTP mix	30" 58°C	
1x *Pfu* reaction buffer	2' 72°C	
0.6 units *Pfu* DNA polymerase	10' 72°C	
ddH_2O up to 50 µl		

Correct size of PCR products was verified on 1% agarose gel, based on 100 bp DNA Ladder. Ladders and samples were always mixed 5:1 with loading dye before loading on gel and all gels were scanned with Typhoon 8600 Variable Mode Imager. The bands of proper size were excised from gel and purified using QIAquick Gel Extraction Kit, after which some of the purified product was run on 1% agarose gel together with Low DNA Mass™ Ladder so that their concentrations could be estimated. This was necessary for the second PCR step of R77C and D112G and for cloning of all final PCR products, which is the subject of the subsequent chapter.

3.1.2 Construction of pcDNA3.1-GH

Purified PCR product was subcloned into the expression vector pcDNA3.1 V5-His-TOPO – briefly pcDNA3.1 – using pcDNA™3.1 Directional TOPO® Expression Kit and following its instructions. The principle behind directional TOPO cloning is the use of topoisomerases and a GTGG recognition sequence, which guarantees correct insert orientation. The vector and its recognition sequence are shown in figure 14. Positive clones would be ampicillin-resistant so the transformed *Escherichia coli* (*E. coli*) TOP10 bacteria were grown on LB/amp plates o/n at 37°C in an Orbital Shaker Incubator without shaking. On the following day some of the colonies were chosen for amplification in 3.8 ml LB/amp medium and cultured as the plates but with 150 rpm. 0.8 ml of each culture was used for a glycerol stock, hence mixed 4:1 with 80% glycerol and stored in -80°C. Plasmids were isolated from the remaining bacteria with QIAprep Spin Miniprep kit.

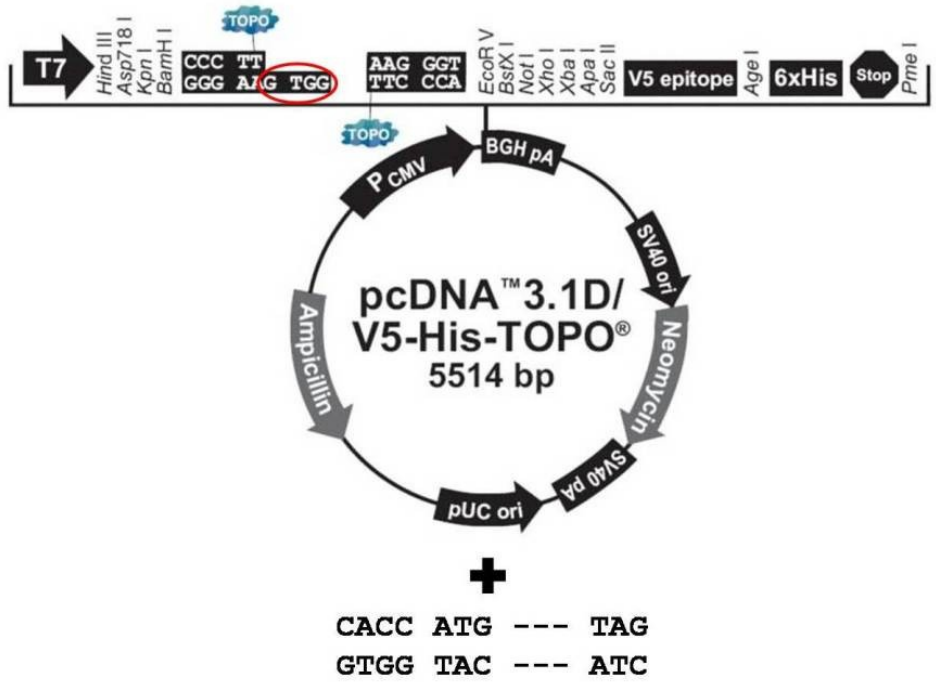

Figure 14: *Structure of the directional TOPO cloning vector.* The GTGG of the insert is cleaved off while the GTGG of the vector (red circle) becomes part of the plasmid. The figure was modified from the pcDNA3.1 Directional TOPO® Expression Kit -manual.

The obtained plasmids, i.e. the "minipreps", were digested with *Bam*HI & *Bgl*II in REact®3 buffer and with *Hind*III & *Eco*RV in REact®2 buffer for further analysis.

Restriction analysis composition

5 µl miniprep	
2 µl 10x REact® buffer	→ 1-2 h at 37°C
0.5 µl = 5 units enzyme I	+ 4 µl 6x loading dye
0.5 µl = 5 units enzyme II	→ 10 µl on 1% agarose gel
autoclaved ddH_2O up to 20 µl	

Restriction maps had been created with Redasoft Visual Cloning software, and a simplified version is presented in figure 15. Restriction sites of none of the used enzymes were lost by introducing the mutations mentioned in chapter 3.1.1 but the deletion mutation Δ188-190 reduced plasmid size and *Eco*RV restriction site by 9 bp.

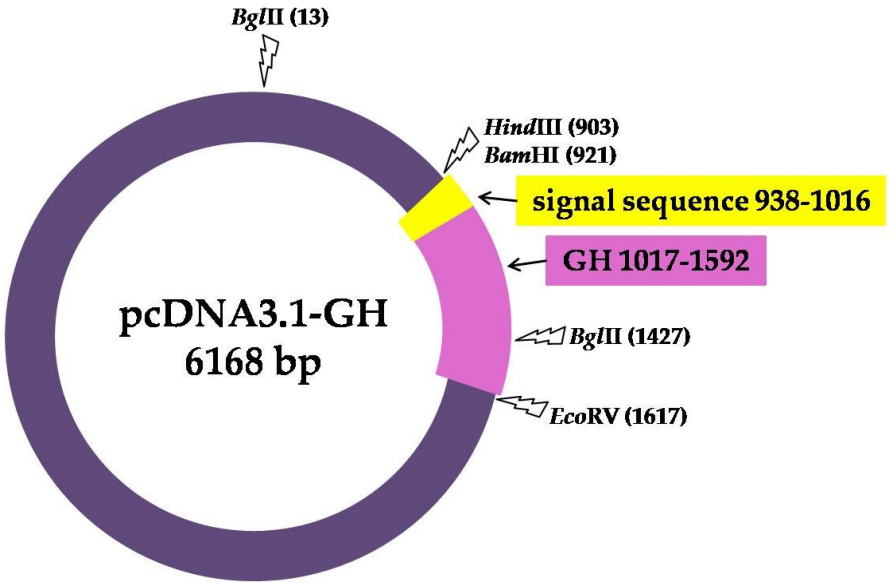

Figure 15: *Restriction map of pCDNA3.1-GH (wt).* Restriction sites are based on analysis with Redasoft Visual Cloning. By digesting the plasmid with *Bam*HI and *Bgl*II, which has one restriction site within the insert and another in vector, one can verify the presence and correct orientation of insert. *Eco*RV and *Hind*III cut on vector on both sides of insert and thus give information about insert size.

For each mutant and for wt GH, one clone that showed correct structure in restriction analysis, based on 100 bp and 1 kb DNA ladders, was chosen for large-scale amplification. Firstly LB/amp plates were inoculated with the glycerol stocks and incubated o/n at 37°C. In the following morning 2-3 ml o/d cultures with a single colony from each plate were started and these were transferred to 100 ml o/n cultures in the evening. HiSpeed® Plasmid MIDI kit was used for plasmid isolation and TE buffer for DNA elution. Finally correctness of constructs was verified by sequencing the plasmids with the standard primers T7 and BGH-rev at IIT Biotech (Bielefeld, Germany). With these plasmids one can sequence the error prone junctions between insert and vector. Also these primer sequences are listed in appendix IV, and in addition their sites are indicated on the vector sequence in appendix V.

3.2 Cell culture

A few different cell lines were employed during the characterization of the mutants. All of them were cultured in 75 cm^2 flasks at 37°C in a CO_2 incubator, in the presence of 10% CO_2. Their culture media are listed in table 5.

Human embryonic kidney (HEK)-293 cells, received from Heike Biebermann (Berlin, Germany), were used for production of recombinant GH. HEK-293 cells stably transfected with hGHR, i.e. HEK-293-GHR cells, were a gift from Richard Ross (Sheffield, UK). HEK-293-GHR cells were used for STAT5 transcription assay and GHR binding assay. Both HEK-293 and -GHR cell lines were passaged 1:4-7 every 3-4 days by rinsing with PBS, trypsinising and resuspending in fresh culture medium.

BaF-B03-B2B2 cells, which also are stably transfected with hGHR, had been a gift from Mauro Bozzola (Pavia, Italy). They were used in the BaF-B03 proliferation assay. BaF-B03-B2B2 cells grow in suspension and were maintained by diluting them 1:10-20 every 2-3 days.

Whenever cells were needed for an experiment or for transfection, their cell concentration had to be determined. For this purpose some of the cells were mixed with 0.4% Trypan blue stain, which stains dead cells, and counted on a Neubauer counting chamber.

Table 5: *Composition of culture media and other solutions needed for cell culture.* Everything was kept sterile.

BaF-B03 culture medium	RPMI-1640 containing ✓ 10% foetal calf serum (FCS), 10% horse serum (HS) ✓ 100 U/ml penicillin, 100 µg/ml streptomycin ✓ 4 mM L-glutamine ✓ 100 ng/ml recombinant hGH (rhGH, IRP 88/624)
Geneticin, 50 mg/ml	✓ 1 g geneticin in 20 ml sterile ddH_2O ✓ filtrated with a 0.2 µm filter ✓ stored in -20°C
HEK-293 culture medium	DMEM containing ✓ 10% FCS ✓ 50 µg/ml Gentamicin ✓ 4 mM L-glutamine, 1 mM pyruvate
HEK-293-GHR culture medium	DMEM:F12 containing ✓ 10% FCS ✓ 100 U/ml penicillin and 100 µg/ml streptomycin ✓ 2 mM L-glutamine ✓ 15 mM HEPES ✓ 400 µg/ml geneticin
PBS	✓ 1 PBS tablet per 500 ml ddH_2O ✓ autoclaved ✓ stored at 4°C

3.3 Production of recombinant GH

HEK-293 cells were used for production of wt and mutant GH. The cells were transiently transfected with the pcDNA3.1-GH constructs and consequently, due to the signal sequence, GH was secreted in the medium.

The medium was collected and presence of GH was verified by Western blot and time-resolved immunofluorometric assays (TR-IFMAs). As soon as GH production could be confirmed, stable transfections were done in order to obtain wt or mutant GH producing cell lines.

3.3.1 Transfection

HEK-293 cells were transfected with the pcDNA3.1-GH constructs, using Lipofectamine™ 2000 as transfection reagent. The cells were seeded on the day before transfection according to table 6. In the same table are summarized the needed amounts of media, DNA and lipofectamine for different culture dish formats. Both DNA and lipofectamine were diluted in Opti-MEM® I Reduced Serum Medium. Diluted lipofectamine was incubated for 5 min at RT before mixing it with the diluted DNA. The mixture was then incubated for 20 min at RT, after which it was dropwise pipetted onto the cells. 24 h post transfection the medium was changed into Biowhittaker® Pro293a-CDM medium supplemented with 4 mM L-glutamine, 1 mM pyruvate and 50 µg/ml Gentamicin. The medium, where GH was being secreted, was harvested 3 days later for experiments. Medium from nontransfected cells was used as negative control in bioassays.

Table 6: *Transfection with Lipofectamine™ 2000.*

cell culture dish	cells	plating medium	DNA in media volume	Lipofectamine in media volume
24-well plate	$1x10^5$	0.5 ml	0.8 µg in 50 µl	2 µl in 50 µl
12-well plate	$2x10^5$	1 ml	1.6 µg in 100 µl	4 µl in 100 µl
6-well plate	$5x10^5$	2 ml	4 µg in 250 µl	10 µl in 250 µl
75 cm^2 flask	$2x10^6$	10 ml	8 µg in 500 µl	20 µl in 500 µl

3.3.1.1 Creation of stable cell lines

HEK-293 cells on a 24-well plate were transfected with wt or mutant GH-containing plasmids as described above. After 24 h the cells were passaged

1:10 and on the following day 0-1000 μg/ml geneticin was added per well. The neomycin gene that the pcDNA3.1 plasmid carries (see figure 14) provides resistance against the antibiotic geneticin, also known as G418, in eukaryotic cells [Wagman, 1980]. Once selection started to show, individual colonies were picked and seeded on a 24-well plate. The cells were further cultivated in presence of 1000 μg/ml geneticin and finally the cells producing highest amount of recombinant GH were chosen for larger cultures with reduced geneticin concentration (500 μg/ml).

3.3.2 Separation of Δ188-190 monomer and dimer

Δ188-190 was expressed mainly as a mixture of monomer and dimer. In order to do experiments with exclusively dimer, dimer and monomer were separated. This was done by Dr. Zida Wu, but here is a brief description. A large amount of the mutant GH was produced by culturing the Δ188-190-expressing HEK-293 cell line in a miniPERM cell culture module. The mutant-containing supernatant was concentrated with Roti-Spin MINI 3 columns and then applied into a Superdex 75 column. Dimer and monomer were separated by size exclusion using a FPLC system. PBS was used for elution and 250 μl fractions were collected. Concentrations of the fractions were measured by TR-IFMAs (see chapter 3.3.3.2) and the fractions containing monomer on one hand and dimer on the other were pooled together. Presence of monomer vs. dimer was confirmed in Western blot. Finally the samples were sterile filtered using a 0.2 μm filter.

3.3.3 Confirmation of expression

Cell supernatants from transient transfections or stable cell lines were analysed qualitatively by Western blot and quantitatively by TR-IFMAs. These methods were also used to verify the separation of Δ188-190 monomer and dimer and to measure their concentrations.

3.3.3.1 Western blot

Western blot was done in order to verify that GH is in fact secreted in the growth medium, has the expected size and to see if it's forming dimers or oligomers.

3.3.3.1.1 Procedure

Table 7: *Working solutions for Western blotting.*

SDS-PAGE gel **(enough for two 12% 0.75 mm gels)**	Resolving gel 2.5 ml 3 M Trizma® base, pH 8.8 (HCl) 100 µl 20% SDS (w/v) 4 ml 30% Acrylamide/Bis Solution 3.3 ml ddH_2O 50 µl 10% APS (w/v) 5 µl TEMED Stacking gel 2.5 ml 0.5 M Trizma® base, pH 6.8 (HCl) 50 µl 20% SDS 1.3 ml 30% Acrylamide/Bis Solution 6.09 ml ddH_2O 50 µl 10% APS 10 µl TEMED
SDS-PAGE 10x running buffer	✓ 0.25 M Trizma® base, 1.92 M glycine, 1% SDS, 0.01% NaN_3 ✓ diluted 1:10 before use
Towbin buffer	✓ 25 mM Trizma® base, 192 mM glycine, 20% MeOH in ddH_2O
PBS-Tween	✓ 1 PBS tablet per 500 ml ddH_2O ✓ 0.05% Tween® 20 added

Either reducing or non-reducing Lane Marker Sample Buffer was added to the supernatant and the proteins were separated by 12 % SDS-PAGE. Kaleidoscope Prestained Protein Standard was used as size reference. The samples were first stacked for 15 min at 70 V and then separated for 90

min at 110 V in 1x running buffer. The separated proteins were transferred into a nitrocellulose membrane between sheets of blotting paper, all wetted in Towbin buffer using a Trans-Blot® Semi-Dry Electrophoretic Transfer Cell. Thereafter, in order to prevent subsequent unspecific antibody binding, the membrane was blocked with 5% skim milk in PBS-Tween either o/n at 4°C or for 1 h at RT under agitation on a rocking platform.

After blocking and also later on, after both antibody incubations, the membrane was washed thoroughly with PBS-Tween. As primary antibody a mixture of two in-house monoclonal α-hGH antibodies, 10A7 [Gertler, 1996] and 7B11 [Strasburger, 1996], was used, both diluted to 1µg/ml in 0.5% skim milk/PBS-Tween. Secondary antibody was a sheep-derived, horseradish peroxidase (HRP)-labelled α-mouse antibody, diluted to 0.5 µg/ml in 0.5% skim milk/PBS-Tween. Both incubations were carried out for 1 h at RT under agitation. Finally, the bands were visualized in an enhanced chemiluminescence (ECL) reaction using Amersham ECL™ Western Blotting Detection Reagents. The light reaction was captured on Amersham Hyperfilm and the film was developed using an automated Curix 60 table-top processor.

3.3.3.2 TR-IFMA

TR-IFMAs employing different in-house monoclonal antibodies (mAbs) were used for determination of GH concentration in media. Biotinylated 10A7 (10A7-b) was always used as tracer antibody (mAb2-b in figure 16) and three different mAbs, named 7B11, 8B11, 6C1 [Thorner, 1999] and 1B3 [unpublished], were used as capture antibodies (mAb1 in figure 16) depending on the mutation in question. 7B11, 8B11 and 6C1 gave an approximately same concentration for wt GH. 7B11 or 8B11 was used when C182A, C189A and C182+9A were measured. 8B11 was used for R77C and D112G because 7B11 appeared to bind particularly strongly to D112G giving a false concentration. 6C1 was used for Δ188-190 and its monomeric

and dimeric forms since it can equally measure dimer and monomer. 1B3 recognises preferentially dimeric GH and was therefore used to measure the degree of dimerization. Binding epitopes of the used mAbs except for 1B3 can be found in appendix VI [Wu, 1998]. Generation and biotinylation of antibodies had been done by Dr. Zida Wu as described by Strasburger et al. [1996]. The hGH standard used in this assay was pituitary hGH (phGH; IRP 80/505) which best resembles GH secreted by eukaryotic cells [Jansson, 1997].

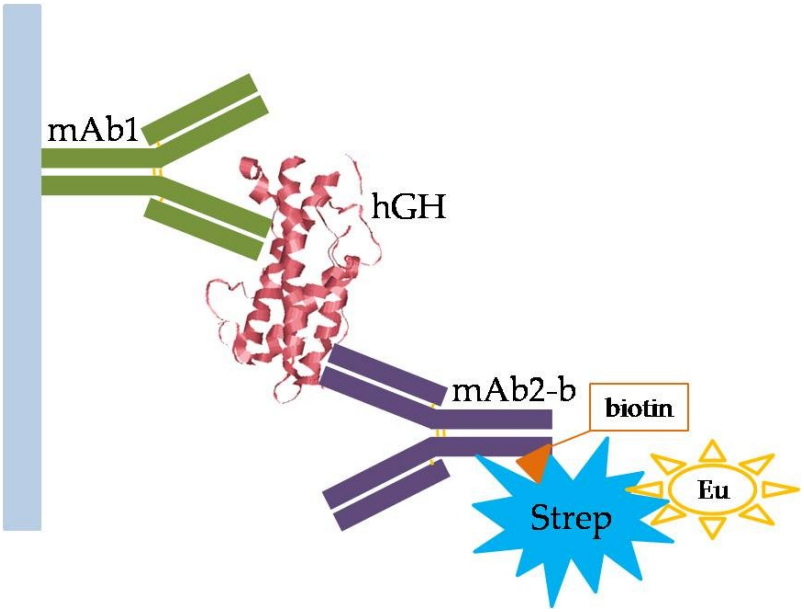

Figure 16: *TR-IFMA.* mAb1, which can be either 7B11, 8B11, 6C1 or 1B3, captures the GH (here in a random posture), to which the biotinylated tracer antibody 10A7-b binds. Europium (Eu)-labelled streptavidin (Strep) binds to the biotin, and the europium can be measured in dissociation-enhanced time-resolved fluorometry.

3.3.3.2.1 Procedure

Table 8: *Working solutions for TR-IFMA.*

Assay buffer	✓ 50 mM Trizma® base, 154 mM NaCl, 20 µM DTPA, 0.01% Tween 40, 0.05% NaN_3, 0.05% bovine γ-globulin and 0.5% BSA in ddH_2O ✓ pH 7.75 ✓ filtration with 8 µm cellulose nitrate filter ✓ storage at 4°C

Coating buffer	✓ 50 mM Na_2HPO_4 in ddH_2O ✓ pH 7.4
Enhancement solution	<u>100x Enhancement solution I</u> ✓ 100 mM thenoyltrifluoroacetone, 10 mM tri-n-octylphosphinoxide, 2% EtOH and 20% Triton® X-100 in ddH_2O ✓ storage at 4°C <u>10x Enhancement solution II</u> ✓ 68 mM KHP and 6% HAc in ddH_2O ✓ storage at 4°C <u>Working solution</u> ✓ 1 % Enhancement solution I mixed with 10 % Enhancement solution II

A 96-well flat bottom microtiter plate was coated with 500 ng mAb1 in 200 µl coating buffer per well, covered with self-adhesive film and stored in 4°C for a minimum of 12 h. The plate was washed with PBS-Tween after every incubation step, using a Columbus 12 Channel Head 96 well Strip Plate Washer. Onto the mAb1-coated plate, 175 µl assay buffer per well was pipetted. To this, 25 µl phGH with increasing concentrations (0 to 100 ng/ml) or 25 µl sample, both diluted in assay buffer, was added and the plate was incubated for 2 h. In all incubation steps of this and other immunoassays the plate was agitated on a DELFIA® Plate Shaker at RT if not otherwise specified. A subsequent 2 h incubation was done with 50 ng biotin-labelled mAb 10A7 (10A7-b=mAb2-b in figure 16) in 200 µl assay buffer per well. In the following steps the plate was first incubated with europium-labelled streptavidin (streptavidin-Eu) and then with enhancement solution as described in figure 17. Fluorescence signal was read on VICTOR3™ Multilabel Counter. Based on the phGH standard curve, concentrations of samples were calculated automatically by Multicalc program.

Figure 17: *Finishing a TR-IFMA.* This procedure was used in all fluorometric assays of this project. The plate was first incubated for 30 min with 20 ng streptavidin-Eu in 200 µl assay buffer per well then for 10 min with 200 µl enhancement solution per well. In the competitive GHBP binding assay (chapter 3.4.1.2) the amounts of streptavidin-Eu, assay buffer and enhancement solution were halved, i.e. 10 ng streptavidin-Eu, 100 µl assay buffer and 100 µl enhancement solution per well. Finally the fluorescence was read on VICTOR3™ Multilabel Counter.

3.4 Functional and structural characterization of recombinant GH

The supernatants with known GH concentrations were used for an array of experiments in order to gain information about the binding affinity, biological activity and stability of the different mutant GHs in comparison to wt GH.

3.4.1 Binding studies

First of all the binding affinity was investigated with two immunoassays employing GHBP. For studies with full-length hGHR, the HEK-293-GHR cell line and radioactive ^{125}I-hGH were utilized.

3.4.1.1 Immunofunctional assay

With this sandwich assay the functionality of both binding sites of the mutants compared to wt hGH could be studied. The assay has been developed by Strasburger et al. [1996 & 1999]. 7B11, which recognizes binding site 2 of hGH, is used as capture antibody and biotin-labelled recombinant hGHBP (GHBP-b), which in this case binds to binding site 1, as tracer. Unlike in the TR-IFMAs, rhGH (IRP 88/624) was used as standard. The assay is elucidated in figure 18.

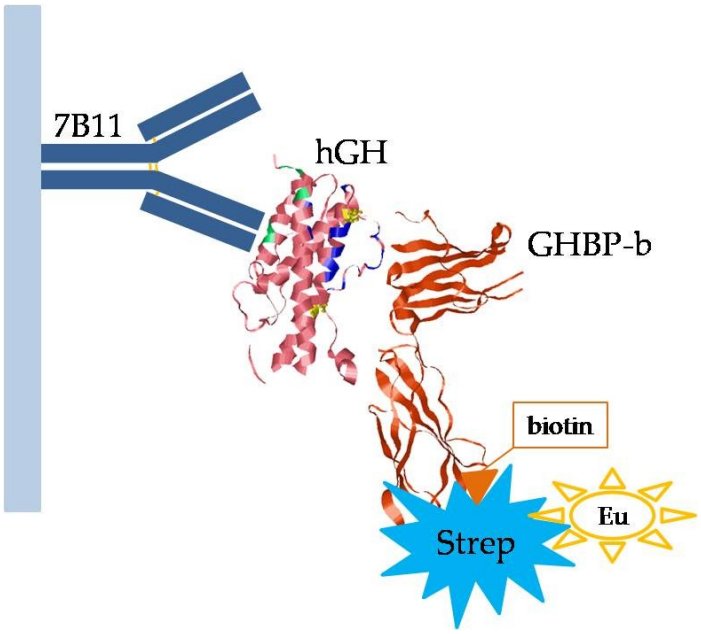

Figure 18: *Immunofunctional assay.* The assay scheme is similar to the one described in figure 10. The green binding site 2 of GH is recognized by the mAb 7B11 and the blue binding site 1 binds to a biotinylated GHBP molecule.

3.4.1.1.1 Procedure

The plate was coated with 500 ng mAb 7B11 in 200 µl coating buffer per well, just like in the TR-IFMA (chapter 3.3.3.2). After a wash step, 175 µl assay buffer and 25 µl sample or standard was added per well and the plate was incubated for 3 h. Incubation with 25 ng GHBP-b in 200 µl assay buffer per well took place o/n at 4°C. GHBP had been a gift from Professor Gertler (Rehovot, Israel) and it was biotinylated by Dr. Zida Wu as described by Strasburger et al. [1996]. The assay was finished as described in figure 17. The immunofunctional concentration was calculated by the Multicalc program based on rhGH standard curve. An immunofunctional proportion was obtained by comparing this immunofunctional concentration to the one measured by TR-IFMA.

3.4.1.2 Competitive GHBP binding assay

Cross-reactivity is an indicator of binding affinity. Thus a competitive assay where labelled GHBP bound to GH is displaced by increasing concentrations of unlabelled GHBP gives a good idea of GH's binding affinity to GHBP. Since GHBP equals to the extracellular domain of full-length receptor, one can from this assay also quite well predict GH's binding affinity to GHR.

First step was to determine a concentration for each sample where the obtained fluorescence would correspond to approximately 10% of maximal fluorescence measured with wt GH. A sandwich assay employing mAb 10A7 and GHBP-b was used for this purpose. 10A7 recognises an epitope far from both binding sites and thus leaves them available for GHBP. In the subsequent assay GHBP and GHBP-b compete for 10A7-bound GH. The assay is illustrated in figure 19.

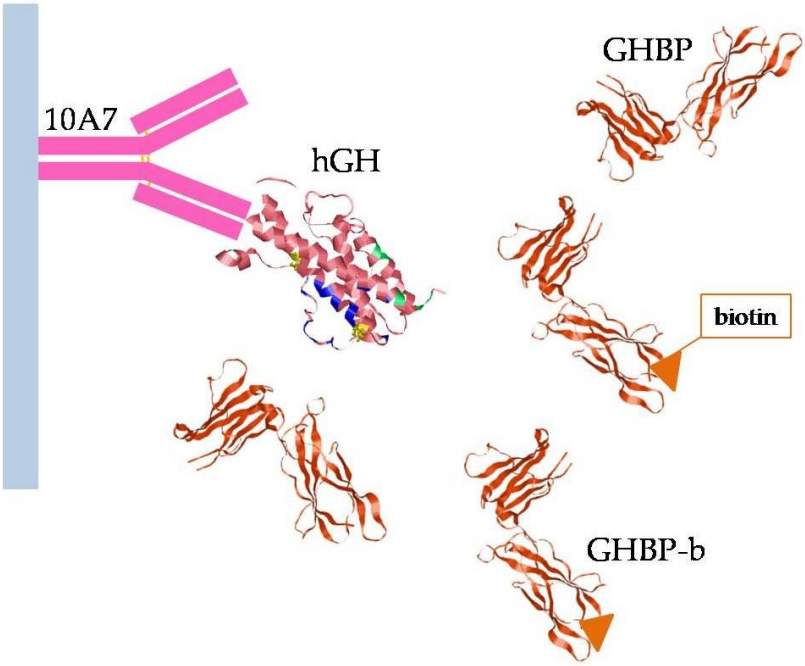

Figure 19: *GHBP binding assay.* In the pre-assay only GHBP-b is let bind to GH but in the final assay GHBP-b competes with increasing concentrations of unlabelled GHBP. Streptavidin-Eu is again used for detection.

3.4.1.2.1 Procedure – step 1

Same solutions as in TR-IFMA were used (see table 8). 75 µl assay buffer and 25 µl sample were pipetted to the wells of a 96-well plate coated with 500 ng mAb 10A7 in 100 µl coating buffer per well. Sample was either 0 to 1000 ng/ml wt GH for the definition of maximal signal or 0 to 100 ng/ml GH in cell supernatant for definition of the 10% maximum. All samples were diluted in assay buffer. After incubating the plate for 2 h with the samples, 10 ng GHBP-b in 100 µl assay buffer per well followed. After another 2 h incubation the assay was finished as in figure 17, but with halved amounts. Fluorescent counts were plotted against hGH concentration (MS Office Excel) and the concentration at 10% of maximal counts measured with wt GH was calculated for each sample. A trend line was inserted for the linear part of the curve and the appropriate concentration was calculated with the formula of the trend line.

3.4.1.2.2 Procedure – step 2

Onto a 96-well plate coated with 10A7 as in step 1, 75 µl assay buffer and 25 µl sample with the concentration calculated above were pipetted, two rows per sample. After incubating for 2 h, GHBP and GHBP-b followed. First 50 µl 0 to 1000 ng/ml unlabelled GHBP (diluted in assay buffer) was pipetted to each pair of rows, and then 10 ng GHBP-b in 50 µl assay buffer was added to all wells. The assay went on as described in figure 17, but with half the amounts of buffer and streptavidin-Eu. Acquired counts were divided by the counts of the same GH molecule at 0 ng/ml GHBP, and this so called B/B0 value was plotted against the increasing GHBP concentration, whereby a sigmoidal curve was obtained. The curve was analysed with GraphPad Prism 5.01 by nonlinear regression using the sigmoidal dose-response equation. The greater the concentration of unlabelled GHBP required for displacement of 50% of GHBP-b, i.e. the IC50 value, the lower the binding affinity of the GH molecule in question.

3.4.1.3 GHR binding assay

In order to examine GH binding to full-length receptor, an assay exploiting ^{125}I-labelled GH and HEK-293 cells stably transfected with full-length human GHR (HEK-293-GHR) was performed. Similarly, as in the previous experiment, the biotin-labelled GHBP bound to GH was displaced by increasing concentrations of unlabelled GHBP, here radioactively labelled GH is displaced by increasing concentrations of sample GH on a monolayer of GHR-expressing cells.

3.4.1.3.1 Procedure

Table 9: *Binding buffer for GHR binding assay.*

Binding buffer	4x stock: ✓ 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.3343 mM $Na_2HPO_4x12H_2O$, 5.55 mM glucose ✓ pH 7.4 Working solution: ✓ 1.3 mM $CaCl_2$, 280 mM sucrose, 0.2% BSA and 2.5% skim milk in 1x stock solution

75,000 HEK-293-GHR cells per well on a 48-well plate were seeded and let attach o/n. On the following day the cells were first washed with binding buffer. Then 100 µl of 0 to 20 nM GH diluted in binding buffer and 60,000 cpm of ^{125}I-hGH in 100 µl binding buffer were added to each well and the plate was again incubated o/n. To finish the assay, the cells were washed twice with ice cold binding buffer after which 500 µl 0.1 M NaOH was added and counts were measured. The higher the counts were, the less sample GH had bound to the cells and thus the lower its binding affinity to GHR was. The counts of each sample were divided by counts at 0 nM GH. These B/B0 values were plotted against GH concentration and IC50

values based on fitted sigmoidal dose-response curves were calculated with GraphPad.

3.4.2 Bioassays

Biological activity of the mutants was studied by two assays – BaF-B03 assay measuring their proliferative potential and STAT5 assay determining their ability to initiate signal transduction. In order to rule out any effect of the cell supernatant per se, a sample from cells that had been "transfected" without plasmid was included as control. It was diluted as the GH sample with lowest GH concentration, i.e. with highest content of serum-free medium.

3.4.2.1 BaF-B03 proliferation assay

BaF-B03 is a mouse IL3-dependent pro-B cell line isolated from the parental Ba/F3 cells [Hatakeyama, 1989; Palacios, 1985]. Behncken et al. [1997] were the first to stably transfect these cells with hGHR for their GH binding studies. Rowland et al. [2002] used identical cells, naming them clone B2B2, for a proliferation assay measuring GH bioactivity. BaF-B03-B2B2 cells can be maintained with hGH instead of IL3. Proliferation assays for hGH have also been applied by Yoshizato et al. [2000] and Ishikawa et al. [2000] but they used the parental Ba/F3 cells instead of the subclone BaF-B03. We used the assay developed by Rowland et al. [2002] as basis for our proliferation assay.

3.4.2.1.1 Procedure

Table 10: *Starvation medium for BaF-B03 proliferation assay.*

BaF-B03 starvation medium	RPMI-1640 containing ✓ 0.5% HS ✓ 100 U/ml penicillin, 100 μg/ml streptomycin ✓ 4 mM L-glutamine

The BaF-B03-B2B2 cells were cultivated as described in chapter 3.2. Once cell density was about 50,000-100,000 cells/ml, the cells were washed with PBS and starved o/n in growth medium without rhGH and FCS and only 0.5% HS (starvation medium). On the following day, the cells were again washed with PBS, and then 25,000 cells in 50 µl starvation medium per well were pipetted on a white 96-well flat bottom cell culture plate. Wt and mutant hGH were diluted in starvation medium and added to the wells as additional 50 µl. The plate was incubated for 24 h, after which the cells were lysed and their ATP content was measured using the ViaLight® Plus Kit. The method is based on the following reaction:

$$\mathbf{ATP + Luciferin + O_2} \xrightarrow{\mathbf{Luciferase,\ Mg^{2+}}} \mathbf{Oxyluciferin + AMP + PP_i + CO_2 + light}$$

The obtained luminescence was measured on VICTOR3™ Multilabel Counter. ATP concentration is considered to be a reliable reference for cell number [Dexter, 2003]. In order to be able to compare results from separate experiments with each other, the luminescence of each sample was divided with luminescence of wt at 5 nM. This "total of wt(max)" was plotted against GH concentration and EC50 values for wt and mutants were calculated based on a fitted sigmoidal dose-response curve using GraphPad.

3.4.2.2 STAT5 transcription assay

STAT5 transcription assay is a tool to quantify activation of the Jak/STAT signalling pathway by GH (see chapter 1.1.6.2.1). This is achieved by transfecting HEK-293-GHR cells with a plasmid containing a lactogenic hormone responsive element (LHRE) fused to a minimal thymidine kinase (TK) promoter and firefly luciferase. STAT5 binding to LHRE leads to synthesis of luciferase which can be detected as the amount of substrate that is converted to product in a light-producing reaction. Dexamethasone is added to the stimulation medium because it enhances GH-induced activa-

tion of LHRE [von Laue, 2000]. In order to take transfection efficacy into account, a second plasmid containing the gene for *renilla* luciferase under the control of a constitutively active cytomegalovirus (CMV)-promoter was included in the transfection. Since these two luciferases have different substrates, the luminescence they produce can be detected in two separate reactions and the firefly luciferase activity can be normalised to *renilla* luciferase activity. This was done using the Dual-Luciferase® Reporter (DLR™) Assay System. The reactions catalysed by the firefly and *renilla* luciferases are shown in figure 20.

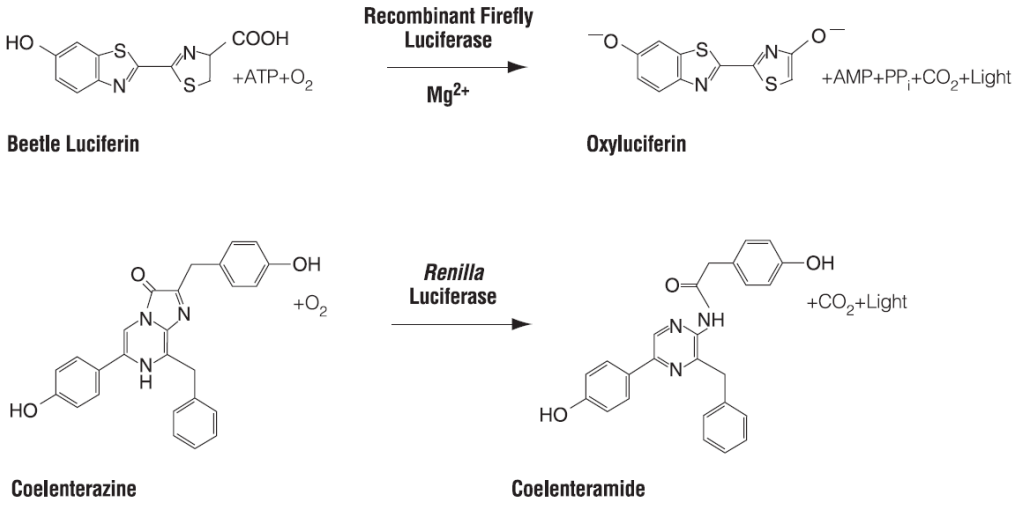

Figure 20: *Reactions catalysed by firefly and renilla luciferases with beetle luciferin and coelenterazine as respective substrates.* Assay reagents contain the substrates whereas the luciferases are encoded by the plasmids, with which the cells have been transfected [Promega's Dual-Luciferase® Reporter 1000 Assay System, Technical Manual].

STAT5 transcription assay was developed by Ross et al. and the first version was published in 1997. They further developed the assay over years [Maamra 1999, Ross 2001] and kindly provided us the needed cells and plasmids. I adapted the assay from 6-well to 96-well format, changed transfection reagent from calsium phosphate to Lipofectamine™ 2000 and increased relative LHRE concentration fivefold for higher signal.

3.4.2.2.1 Procedure

Table 11: *Media for STAT5 transcription assay.*

Rich medium	✓ 1 part HEK-293 culture medium mixed with 2 parts HEK-293-GHR culture medium
Stimulation medium	DMEM:F12 containing ✓ 100 U/ml penicillin and 100 µg/ml streptomycin ✓ 2 mM L-glutamine ✓ 15 mM HEPES ✓ 100 ng/ml dexamethasone ✓ 1% BSA

A ca 80% confluent flask of HEK-293-GHR cells was incubated for 6 h in rich medium. After this, 15,000 cells in 100 µl rich medium per well were seeded on a white 96-well flat bottom cell culture plate. The cells were then transiently transfected with LHRE/TK-luciferase reporter gene construct and phRL-CMV plasmid.

DNA mix	**Transfection mix**
38 ng LHRE/TK-luciferase reporter gene construct 150 pg phRL-CMV 92 ng salmon sperm (for 130 ng total DNA) Opti-MEM® up to 25 µl	Lipofectamine 2000 diluted 1:50 → 5 min at RT 25 µl diluted lipofectamine + 25 µl DNA mix → 20 min at RT

50 µl transfection mix was slowly pipetted into each well of cells. On the following day the cells were stimulated for 6 h with increasing amounts of wt or mutant hGH in 50 µl stimulation medium. Supernatant from cells transfected without plasmid was again used as control in order to rule out the possibility that the medium alone could activate the STAT5 pathway. Finally cells were lysed with 1x Passive Lysis Buffer (DLR™ kit) and their firefly (LHRE) and *Renilla* (phRL-CMV) luciferase activities were meas-

ured with Mithras LB 940 luminometer and MicroWin 2000 software. The course of the DLR™ assay procedure is summarized in figure 21.

Figure 21: *Measurement of the transcription assay.* All steps were done at RT. Luciferase Assay Reagent II (LAR II) and Stop & Glo Reagent were always prepared fresh by mixing substrate with appropriate buffer. Further details can be found in the Technical Manual of the DLR™ Assay System.

Normalised firefly/*Renilla* luciferase activity was further divided by maximal activity of wt/rhGH so that the separate experiments could be compared with each other despite of the differences in minimal and maximal activities between assays. The obtained "total of wt/rhGH(max)" values were plotted against GH concentration using GraphPad.

3.4.2.3 Inhibitions

Antagonistic effect of Δ188-190 dimer and monomer was studied in both bioassays. In that case the sample was 25 µl rhGH with a constant concentration and 25 µl Δ188-190 dimer or monomer with an increasing concentration. B2036 was used as a positive inhibition control. B2036 is the GHR antagonist Pegvisomant without PEG-residues. The obtained luminescence counts from BaF-B03 assay and normalised *Renilla*/firefly luciferase activities were divided by the values obtained with only rhGH. These B/B0 values were plotted against the molar ratio of antagonist and rhGH (GraphPad).

3.4.3 Stability studies

Both methods applied for stability studies are based on the drop in GH concentration due to exposure to degrading substances – in the first ex-

periment substances naturally present in human serum and in the second one the digestive enzyme trypsin.

3.4.3.1 Incubation in serum

GH-containing supernatants were diluted in human serum with [GH] ≤ 0.2 ng/ml and incubated for 24 or 144 h at 37°C with gentle agitation on Eppendorf Thermomixer 5436. Samples were collected at different time points and stored in -20°C until analysis. GH concentrations were measured by TR-IFMA as described in chapter 3.3.3.2 but the samples and standard phGH were diluted in sheep serum instead of assay buffer. 1B3 and 6C1 were used for dimeric Δ188-190 and 7B11 and 8B11 for other samples. The resulting concentrations were compared to the ones at at t(0).

3.4.3.2 Trypsin digestion

Table 12: *Trypsin solution for stability studies.*

Trypsin, 100 µg/ml	✓ 0.1 mg trypsin dissolved in 1 ml 1 mM HCl ✓ storage in -20°C ✓ working solution 1:100 in 1 mM HCl

Wt and mutant hGH were diluted to 500 ng/ml in the serum-free Biowhittaker® Pro293a-CDM medium, in which they had been harvested as well (see chapter 3.3.1). A 100 µg/ml trypsin stock solution was diluted 1:100 with 1 mM HCl to achieve a 1000 ng/ml dilution. 12 µl of this trypsin dilution or 12 µl 1 mM HCl for negative control was added to 120 µl of the 500 ng/ml GH dilution and the mixture was incubated for 2 h at 37°C with gentle agitation on Eppendorf Thermomixer 5436. Samples were collected at different time points and analysed by Western blot in reducing conditions (see chapter 3.3.3.1).

4 Results

GH mutants R77C, D112G, C182A, C189A, C182+9A and Δ188-190 produced in eukaryotic cells were characterized in comparison to wt GH in an array of *in vitro* experiments. All graphs and statistical analysis were performed with GraphPad Prism 5.01 unless otherwise stated. Observed differences between wt and mutant GH were analysed by unpaired *t*-test with Welch's correction (*p < 0.05, **p < 0.01, ***p < 0.001).

4.1 Construction of expression plasmids

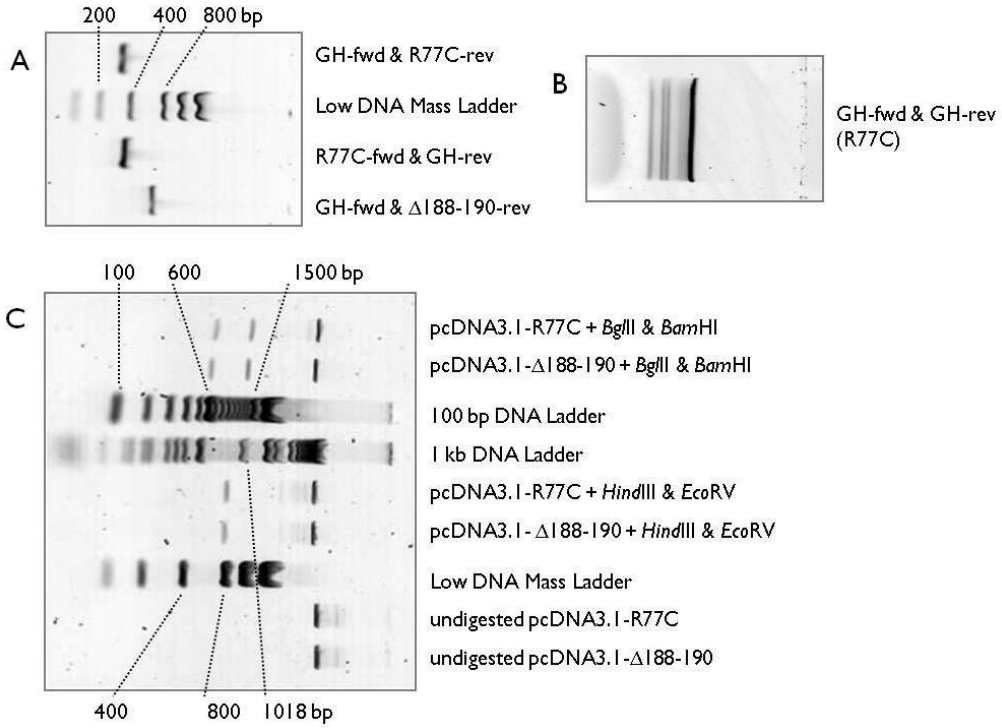

Figure 22: *Construction of expression plasmids.* **A.** Expected sizes of first-step PCR products were 316 bp for GH-fwd & R77C-rev, 365 bp for R77C-fwd & GH-rev, and 649 bp for GH-fwd & Δ188-190-rev. After purification, concentrations of the first PCR products were determined by running 2 µl PCR product and 0.5 µl Low DNA Mass ladder on 1% agarose gel. Estimated concentrations were ~5 ng/µl for GH-fwd & R77C-rev, ~10 ng/µl for R77C-fwd & GH-rev and ~2.5 ng/µl for GH-fwd & Δ188-190-rev (ladders in appendix VII). **B.** Second-step PCR with the GH-fwd & R77C-rev and R77C-fwd & GH-rev fragments using GH-fwd and -rev primers resulted in a 658 bp band and several by-products. The most intense band was purified. **C.** Midipreps pcDNA3.1-R77C and -Δ188-190 were digested with *Bgl*II & *Bam*HI and with *Eco*RV & *Hind*III. Expected sizes with *Bgl*II & *Bam*HI were 506, 908 and 4754/4745 bp (the latter size for Δ188-190), and with *Eco*RV & *Hind*III 714/705 and 5454.

Mutations R77C, D112G, C182A, C189A, C182+9A and Δ188-190 were introduced into the *GH-1* gene by site-directed mutagenesis. In figure 22, R77C is presented as an example for two-step and Δ188-190 for one-step mutagenesis. Fragment sizes of ladders are displayed in appendix VII. The obtained cDNA for wt or mutated *GH-1* was cloned into expression vector pcDNA3.1. Resulting plasmids were first purified in small-scale in order to find correct constructs by restriction analysis with *Bgl*II & *Bam*HI and *Hind*III & *Eco*RV. One of these "minipreps" was then chosen for a larger culture, resulting in "midipreps". The correctness was again checked by restriction analysis (figure 22C) and after that further verified by sequencing.

4.2 Expression of recombinant GH in HEK-293 cells

Recombinant GH was produced by transfecting HEK-293 cells with the midipreps whose correct sequence had been confirmed. GH was secreted in serum-free growth medium, which was used for experiments. GH concentrations were determined by TR-IFMAs. Numerous transfections were done and some typical GH concentrations, usually from 1000 to 2000 ng/ml, can be seen in table 13.

Stable HEK-293 cell lines were created for production of wt and mutant GH. For this purpose the cells were further cultured after transfection and cells carrying the expression plasmid were selected using the antibiotic geneticin. Stable cell lines turned out rather ineffective in GH production, usual TR-IFMA concentrations being 100-300 ng/ml. Because of the higher concentration and also because GH in serum-free medium was preferred for bioassays, GH produced by transient transfection was used for experiments. The Δ188-190 cell line was an exception since it was used for the production of the mutant's monomeric and dimeric forms. For this purpose vast amounts of the Δ188-190 mutant were needed and since during fractionation and purification it was concentrated and the medium

was changed, the low concentration and serum-containing medium were not a problem.

In Western blot the correct size of the recombinant GH was verified and also the concentrations measured by TR-IFMAs were checked for plausibility. Successful separation of Δ188-190-monomer and -dimer could also be seen in Western blot. In figure 23, 2.5 ng mutant or wt GH in either non-reducing or reducing buffer was loaded per lane on 12% SDS-PAGE gel. phGH was included as control.

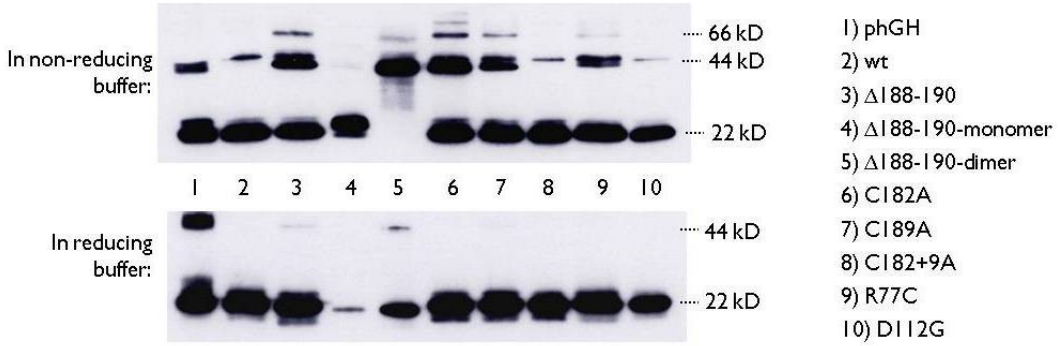

Figure 23: *Western blot.* 2.5 ng of sample in either non-reducing or reducing buffer was blotted. All samples seem to have the correct 22 kD size. Mutants Δ188-190, C182A and C189A with a free C-terminal cysteine form an oligomer, probably trimer, that cannot be seen in the other samples. Some 44 kD dimer is present in all non-reduced samples except for the Δ188-190-monomer. Mutants with a free cysteine are the ones with most dimer. In phGH, Δ188-190 and Δ188-190-dimer some dimer is left even in reducing conditions. Δ188-190-monomer is only present as a faint band in reducing buffer.

Size of recombinant monomeric GH is 22 kD. Sizes and approximate concentrations of wt and all mutants seem correct when compared to phGH and a size standard. In non-reducing buffer Δ188-190-dimer has the correct 44 kD size. Some dimer is present in all samples except for the Δ188-190-monomer. In all samples with a free cysteine, i.e. Δ188-190, C182A, C189A and R77C, also a bigger band – probably a trimer – can be seen. In these mutants also dimer formation is more prominent. Some dimer is left of phGH and, to less extent, of Δ188-190 and Δ188-190-dimer even in reducing buffer. Monomeric Δ188-190 is apparently degraded in reducing buffer and only a faint band is left.

4.3 Binding affinity

Once production of recombinant GH had been confirmed and the GH concentration of supernatants had been quantitatively determined, the actual characterization of the mutants began. One important aspect of GH functionality is its binding to the GH receptor. This was studied by three experiments, two of which were immunoassays based on GH binding to GHBP, which equals the extracellular domain of GHR. In the third experiment binding to full-length GHR on eukaryotic cell surface was examined.

4.3.1 Determination of immunofunctional concentration

A so-called immunofunctional concentration was determined with an immunoassay employing GHBP and the mAb 7B11. A concentration similar to the one measured by TR-IFMA requires that both GH binding sites are unaltered. In this assay rhGH instead of the usual immunoassay standard phGH was used since rhGH contains exclusively the 22 kD isoform of GH molecules fulfilling the prerequisite to be recognised by the combination of mAb 7B11 and GHBP-b. This is the reason why even concentration of wt GH is somewhat lower in the immunofunctional assay when compared to the TR-IFMA – approximately 80%. D112G binds to the mAb 7B11 with higher affinity than wt GH, which explains its high immunofunctional proportion, 97.2% (table 13C). The mutants that showed a much lower immunofunctional proportion than wt GH were Δ188-190 (table 13A), C182A, C189A and C182+9A (table 13B). Their immunofunctional proportions were only 4.1-25.6%. R77C was similar to wt with its 78.2% (table 13C).

Table 13: *Immunofunctional concentrations.* Based on the integrity of both binding sites, an immunofunctional concentration was determined for the GH mutants. Wt measured in the same assay (A-C) was always included in the results. Samples with quite a low immunofunctional proportion are highlighted in the table. These include C182A, C189A, C182+9A and Δ188-190. R77C is similar to wt and, due to high binding affinity to mAb 7B11, D112G has a somewhat higher immunofunctional proportion than wt.

	Sample	TR-IFMA concentration (ng/ml)	Immunofunctional concentration (ng/ml)	Immunofunctional proportion
A	**wt**	1380	1140	**82.6%**
	Δ188-190	1720	70	**4.1%**
B	**wt**	2530	2020	**79.8%**
	C182A	3060	556	**18.2%**
	C189A	2130	163	**7.7%**
	C182+9A	2940	752	**25.6%**
C	**wt**	2590	2040	**78.8%**
	R77C	1040	813	**78.2%**
	D112G	2510	2440	**97.2%**

4.3.2 Binding to GHBP

GH binding to GHBP was studied by an immunoassay, where biotin-labelled and unlabelled GHBP competed for the GH binding sites. GH was first bound to immobilized mAb 10A7, which binds GH far from both binding sites (appendix VI), leaving them exposed for GHBP. Before the competitive GHBP assay was started, the wt GH concentration at which maximal GHBP-b binding was achieved was determined. GH concentration at 10% of this maximum would be used in the competitive assay. This way the starting situation would be the same for wt and each mutant GH. Two maximums, 968,000 and 1,640,000 counts, were determined using two different GHBP-b stocks. In both cases concentration of wt GH was 500 ng/ml wt GH (figure 24A). 100,000 and 160,000 counts, which were approximately 10% of maximum, were chosen as target signals.

In order to determine the concentration at 100,000/160,000 counts for the mutants, 0-100 ng/ml of the GH being tested was assayed and the obtained

counts were plotted against the GH concentration. A trend line was fitted to the curve (not included in figures 24B-D) and the concentration at 100,000/160,000 counts was calculated with its equation.

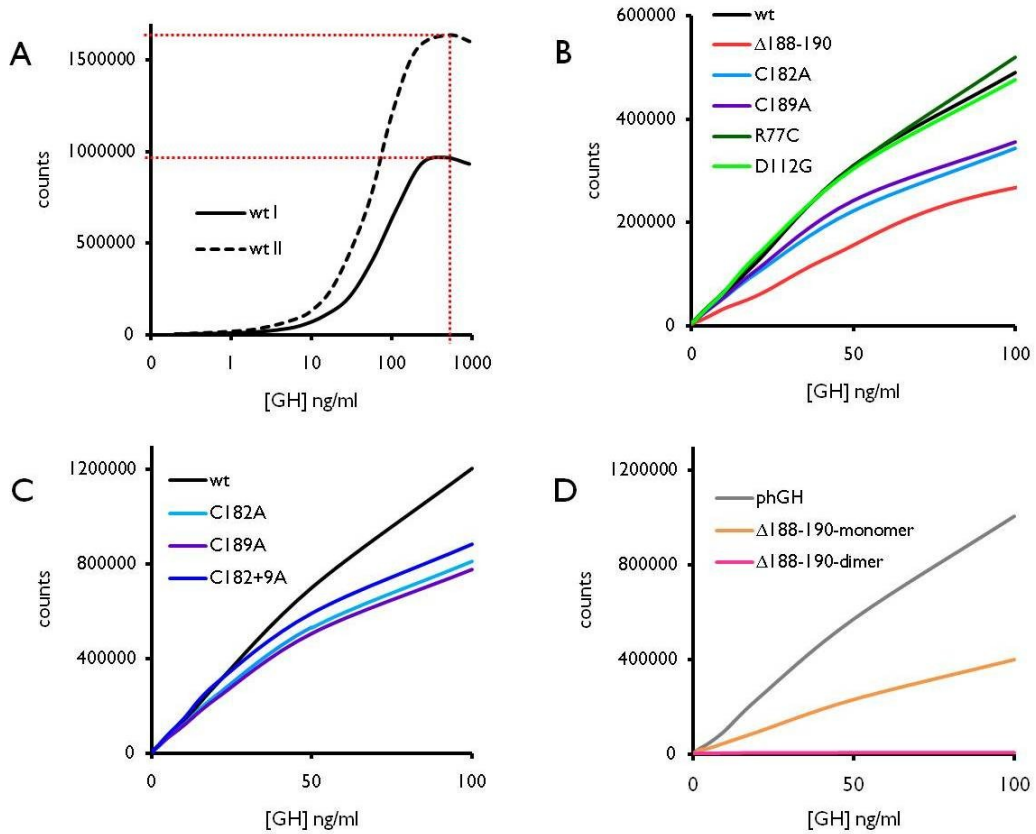

Figure 24: *Competitive GHBP binding assay – step 1.* 10% of maximal binding by wt was determined for each mutant. **A.** As marked with a dashed red line, maximal GHBP-b binding with wt GH was achieved at 500 ng/ml and resulted in 968,000 or 1,640,000 counts, depending on the used GHBP-b stock. Concentration at 100,000/160,000 counts, which was approximately 10% of maximum, was used as assay concentration for wt and mutant GH. **B.** Concentrations at 100,000 counts for wt and the mutants Δ188-190, C182A, C189A, R77C and D112G were 16, 33, 21, 20, 16 and 16 ng/ml, respectively. **C.** When wt, C182A and C189A were compared with the double-mutant C182+9A, the concentrations at 160,000 counts were 13 ng/ml for wt, 17 ng/ml for C182A, 20 ng/ml for C189A and 14 ng/ml for C182+9A. **D.** Δ188-190-monomer and -dimer were compared to phGH. At 160,000 counts the concentration of phGH was 14 ng/ml and of Δ188-190-monomer 35 ng/ml. Δ188-190-dimer bound GHBP very poorly and one would theoretically need 2800 ng/ml of it in order to achieve 160,000 counts.

In figure 24B concentration of wt GH at 100,000 counts was 16 ng/ml. Corresponding concentrations of R77C and D112G were 16 ng/ml as well, but those of C182A and C189A were somewhat higher, both 20 ng/ml. Δ188-

190's concentration at 100,000 counts was 33 ng/ml. Later on when the assay was repeated with C182A and C189A together with C182+9A (figure 24C), the concentrations at 160,000 counts were 13 ng/ml for wt, 17 ng/ml for C182A, 20 ng/ml for C189A and 14 ng/ml for C182+9A. As can be seen in figure 24D, Δ188-190-dimer did not bind to GHBP almost at all. Theoretically, one would have needed a 2800 ng/ml dilution of Δ188-190-dimer to achieve 160,000 counts. This concentration for Δ188-190-monomer was 35 ng/ml and for phGH 14 ng/ml. This preliminary step remained the only result for Δ188-190 dimer and monomer. The concentration at 100,000/160,000 turned out to correlate well with GHBP binding affinity, so one can draw the conclusions that Δ188-190-dimer has an extremely low and Δ188-190-monomer a rather low binding affinity to GHBP in comparison to phGH.

The freshly determined GH concentration at 100,000/160,000 counts was used in the competitive GHBP assay. IC50 values of the mutants were compared to a wt that had been assayed together with them. The obtained curves and IC50 values are presented in figure 25. In case of C182A and C189A, the results in figure B1 were used. The ones in figure B2 were obtained with another GHBP stock, which had quite an influence on the obtained curves and IC50 values.

Four of the mutants, Δ188-190, C182A, C189A and C182+9A, displayed a significantly lower binding affinity to GHBP than wt GH. GHBP binding affinities of R77C and D112G differed slightly from wt (figure 25C) but the standard deviations were rather high, so the differences were not considered meaningful.

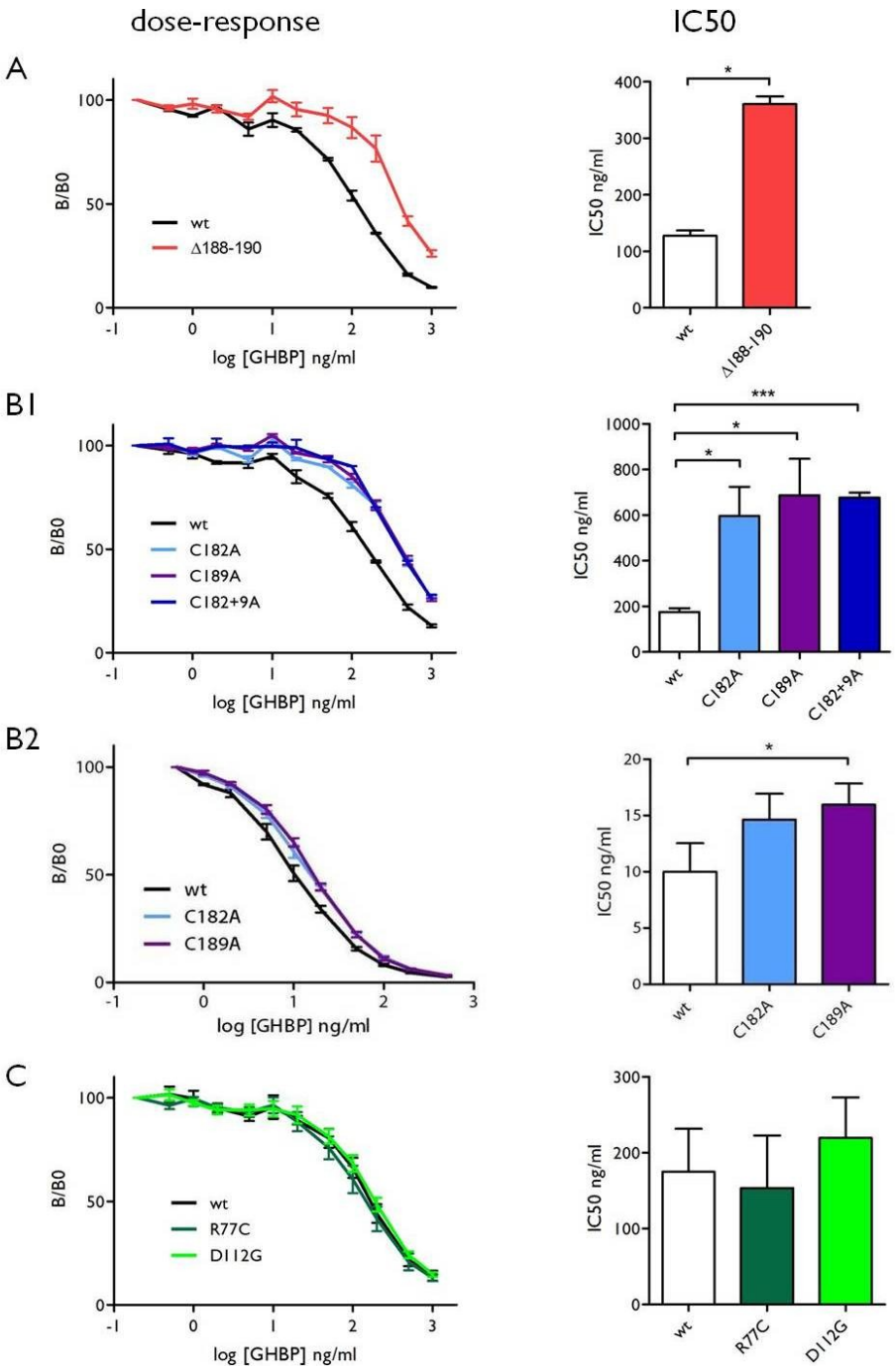

Figure 25: *Competitive GHBP binding assay – step 2.* The concentrations determined in step 1 (figure 24) were used for the competitive assay. B/B0 values were plotted against GHBP concentration. IC50 values were determined based on fitted sigmoidal dose-response curves, which are not included in the figures. **A.** IC50 value of the Δ188-190 mutant was significantly higher than that of wt GH. The data is from two separate experiments. **B1.** C182A, C189A and C182+9A show significantly higher IC50 values than wt GH. **B2.** Due to a different stock of unlabelled GHBP, C182A and C189A seemed more similar to wt – only C189A differed significantly from wt GH. **C.** No significant differences were detected between wt GH and the mutants R77C and D112G. The data is from three separate experiments in B1-C.

4.3.3 Binding to full-length GHR

GH binding to full-length receptor on cell surface was measured by letting increasing amounts of wt or mutant GH compete with a constant concentration of ^{125}I-labelled hGH. HEK-293 cells stably transfected with GHR were used for this purpose. B/B0 values based on the radioactive counts were plotted against GH concentration. Sigmoidal dose-response curves were fitted to the resulting curves and IC50 values were calculated. As shown in figure 26, C182A and C189A have significantly higher IC50 values and thus bind less efficiently to GHR than wt GH. IC50 of Δ188-190 is quite high as well, but the difference is not significant. D112G has a very similar IC50 as wt.

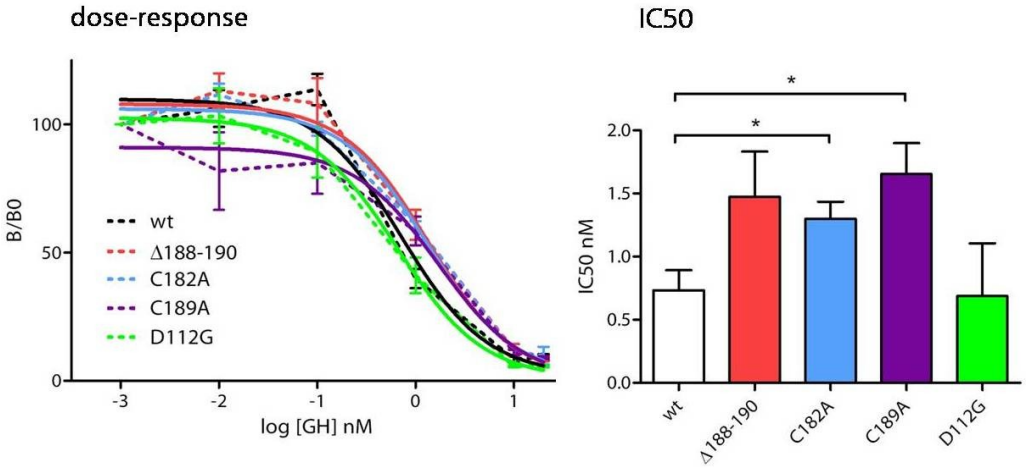

Figure 26: *GHR binding assay.* ^{125}I-labelled GH and increasing concentrations of wt or mutant GH were let bind on HEK-293-GHR cells. B/B0 values were plotted against GH concentration. The dotted curves represent the actual measured values and the continuous ones are fitted sigmoidal dose-response curves. IC50s were calculated and those of the mutants were compared to wt. IC50s of C182A and C189A were significantly higher than that of wt. Δ188-190 had quite a high IC50 as well but the difference was not significant. Data from three separate experiments was used.

4.4 Biological activity

Two approaches were used as the biological activity of the mutants was studied. Their ability to induce cell proliferation was examined in BaF-B03 cells stably transfected with GHR (clone B2B2), and their ability to induce

signal transduction by a STAT5 transcription assay employing HEK-293 cells also stably transfected with GHR. In both assays supernatant from cells transfected without plasmid was used as control in order to ensure that the serum-free growth medium alone did not have the ability to induce cell proliferation or signal transduction.

4.4.1 Proliferative effect

BaF-B03-B2B2 cells were starved o/n and then stimulated for 24 h with wt and mutant GH. Cell count was determined based on ATP content of samples in a luminescent reaction. Induction of proliferation relative to maximal induction by wt GH, the "total of wt(max)", was plotted against GH concentration. Sigmoidal dose-response curves were fitted to the results and EC50 for wt and each mutant was calculated. Results are presented in figure 27. Proliferation induction by the supernatant control remains under 7% and hence the proliferative effect is caused by GH only. One can see in figure 27A that the dose-response curves of Δ188-190 and C189A ascend somewhat later than the others, which are almost upon one another. This difference is even more notable and also statistically significant in the calculated EC50 values (figure 27B). The other mutants did not differ much from wt GH.

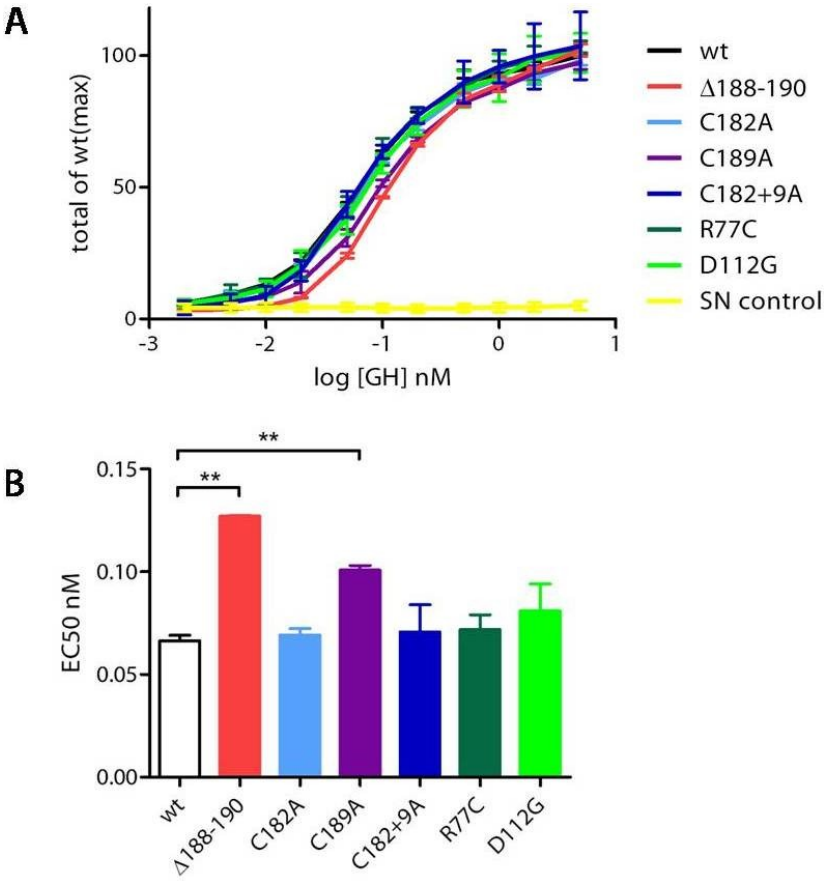

Figure 27: *BaF-B03 proliferation assay.* BaF-B03-B2B2 cells stably transfected with GHR were stimulated for 24 h with wt or mutant GH. Amount of cells was determined by measuring ATP concentration in a luminescent reaction. **A.** Relative stimulation compared to wt(max) was plotted against GH concentration. The supernatant (SN) control had no proliferative effect. Curves of wt, C182A, C182+9A, R77C and D112G are almost one upon each other but the ones of Δ188-190 and C189A ascend slightly later, indicating a lower proliferative effect. **B.** Based on fitted sigmoidal dose-response curves (not included due to similarity to the original curves in 27A), an EC50 value for each mutant was obtained. Two of the mutants, Δ188-190 and C189A, differed significantly from wt. Data from three separate experiments was used for analysis.

Proliferative effects of Δ188-190-monomer and -dimer were compared to the IRP rhGH (produced in *E. coli*) instead of our wt GH produced in HEK-293 cells. Results are shown in figure 28. Much more Δ188-190-monomer than rhGH is required for the same proliferative effect and dimeric Δ188-190 was even less efficient than monomer.

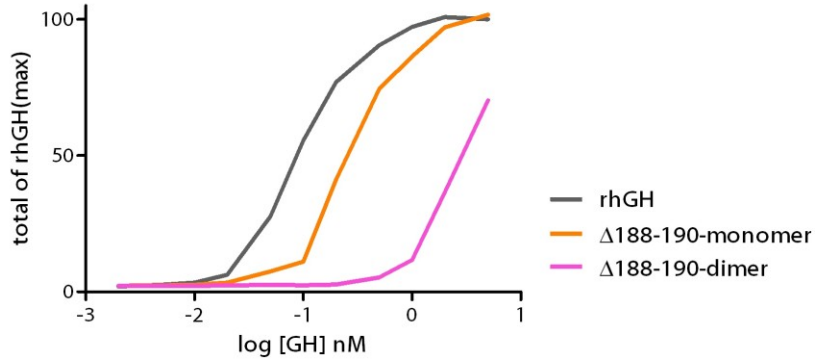

Figure 28: *BaF-B03 proliferation assay with Δ188-190-monomer and -dimer.* BaF-B03 cells expressing GHR were stimulated for 24 with rhGH, Δ188-190-monomer and Δ188-190-dimer. Cell content was determined based on [ATP] in a luminescent reaction. Relative induction compared to rhGH(max) was plotted against GH concentration. Proliferative effect of Δ188-190-monomer is much lower than that of rhGH and that of Δ188-190-dimer is yet lower. The data is from one experiment.

4.4.2 Signal transduction

HEK-293-GHR cells that had been transfected with two gene constructs, one containing a STAT5-activated firefly luciferase gene and the other a constitutively active *Renilla* luciferase gene, were stimulated for 6 h with wt or mutant GH. The cells were lysed and their firefly and *Renilla* luciferase activities were measured. Normalised (firefly/*Renilla*) activity relative to maximal activity of wt was plotted against GH concentration. The activities increase quite linearly so no plateau – and therefore no EC50 values – could be determined. Instead, the relative activities of the mutants were compared to wt at each concentration. Results are presented in figure 29.

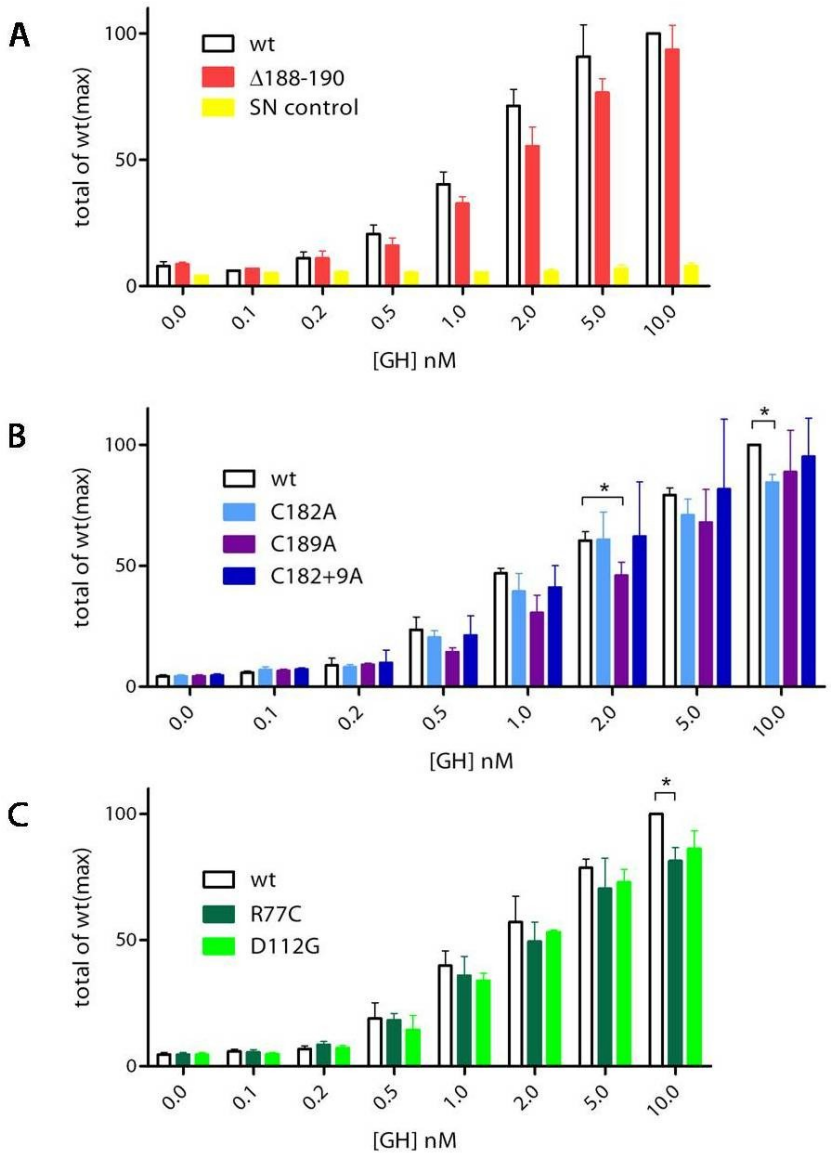

Figure 29: *STAT5 transcription assay.* HEK-293-GHR cells were transfected with a STAT5-activated *Renilla* luciferase construct and a constitutively active firefly luciferase construct. The cells were stimulated for 6 h with wt or mutant GH after which their *Renilla* and firefly luciferase activities were measured. Total normalised (firefly/*Renilla*) activity of wt(max) was plotted against GH concentration. Data of each graph is from three separate experiments. **A.** The SN control was not able to induce STAT5 activation. Δ188-190 shows lower activation than wt especially at 1-5 nM but the differences are not significant. **B.** C189A at 2 nM and C182A at 10 nM show significantly lower STAT5 activation than wt GH. C182+9A is similar to wt. Unfortunately the error bars are quite high especially at higher concentrations. **C.** Both R77C and D112G show a somewhat lower STAT5 activation than wt GH but only the difference between wt and R77C at 10 nM is significant.

As can be seen in figure 29A, the supernatant control did not induce activation of STAT5 pathway. The Δ188-190 mutant seems to be less potent in

activating this pathway than wt GH but the difference is not significant. C182+9A and wt showed similar STAT5 activation whereas that of C182A and C189A was somewhat lower (figure 29B). This difference was significant in case of C189A at 2 nM and C182A at 10 nM. Both R77C and D112G seemed less efficient in activation of STAT5 pathway than wt but only R77C at 10 nM differed significantly from wt (figure 29C).

Δ188-190-monomer and dimer were assayed with rhGH (figure 30). The differences seem much more outstanding than in case of the above mentioned mutants. Dimeric Δ188-190 was not able to activate the STAT5-pathway at all and even the monomeric form was far less potent than rhGH. Dimer at 5 nM and monomer at 10 nM differed significantly from rhGH. 5 nM was the highest concentration of dimer due to low original concentration.

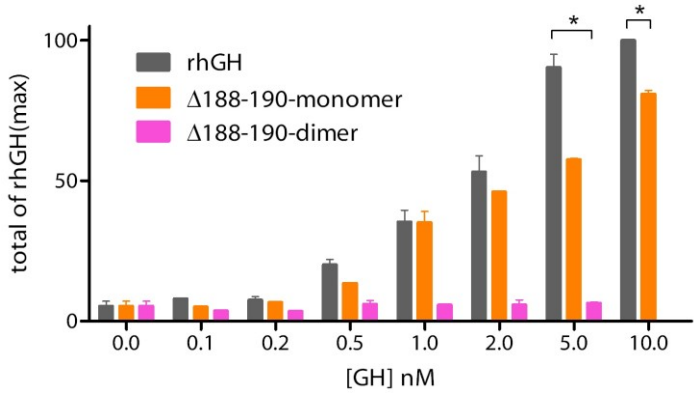

Figure 30: *STAT5 transcription assay with Δ188-190-monomer and -dimer.* HEK-293-GHR cells transfected with a STAT5-dependent *Renilla* luciferase construct and a constitutively active firefly luciferase construct were stimulated for 6 h with rhGH and Δ188-190-monomer and -dimer. *Renilla* and firefly luciferase activities of lysed cells were determined. Normalised (*Renilla*/firefly) activity of each sample was divided by maximal rhGH activity and plotted against GH concentration. Δ188-190-dimer is not able to induce STAT5 activation and the monomer does so to a much lower extent than rhGH. Dimer at 5 nM and monomer at 10 nM differ significantly from rhGH. The data is from two experiments.

4.4.3 Antagonistic effect of Δ188-190

Since it had been hypothesised that the Δ188-190 mutant could be a GHR antagonist, its antagonistic effect was also tested in the bioassays. BaF-B03

proliferation assay and STAT5 transcription assay were completed as described in the previous chapters. B2036, which is Pegvisomant without the PEG-residues, is a known GH antagonist and was used as a "positive" control in both assays. 0.5 or 1 nM rhGH was used in the transcription assay in order to have an as low as possible concentration at which STAT5 activation was prominent (see figure 30). 0.5 nM rhGH was used in the proliferation assay since this concentration had already been established for antagonist assays by others in the group. As can be seen in figure 31, the antagonist effect of B2036 starts to show at 2:1 and is distinct at 20:1 molar ratio in STAT5 assay. In BaF-B03 proliferation assay one needs a 50:1 molar ratio of B2036 in order to slightly reduce proliferation and a 200:1 ratio is required for a more pronounced effect. We could not achieve such ratios with the Δ188-190-monomer and -dimer due to the rather low original concentrations. However, since the molar ratio of dimeric vs monomeric – assumably wt – GH in patients was 3-5:1, going up to a 10:1 molar ratio could have been enough to see if there was any antagonistic effect. Only a 5:1 ratio was achieved with Δ188-190-dimer in the proliferation assay.

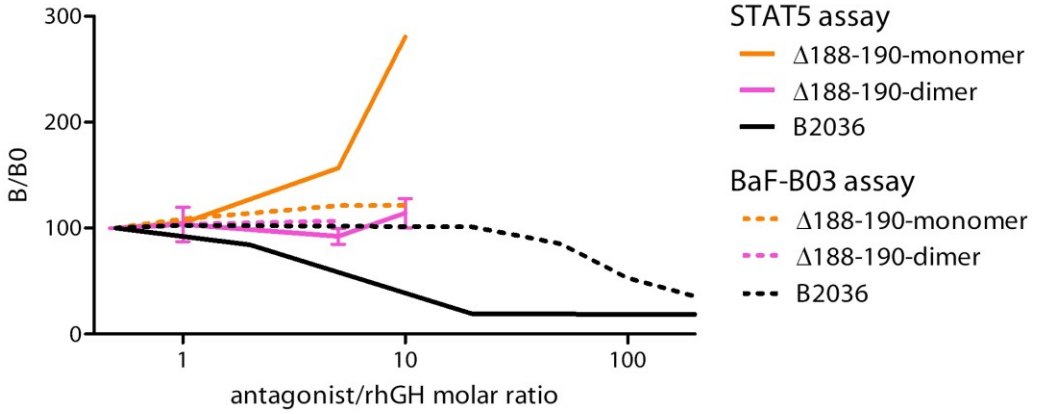

Figure 31: *Antagonistic effect of Δ188-190.* In this graph is combined data from both bioassays, where the antagonistic effects of Δ188-190-monomer and -dimer were studied. Continuous lines represent results from STAT5 transcription assay and the dashed ones from BaF-B03 proliferation assay. 0.5 nM rhGH was used in the proliferation assay and 0.5-1 nM in the transcription assay. B/B0 100 is the value obtained with this rhGH only. The known GH antagonist B2036 was functional in STAT5 assay starting from a 2:1 antagonist:rhGH ratio. In BaF-B03 assay a ratio of 50:1 was required. The dimer was tested up to 5:1 molar ratio in the proliferation assay and 10:1 in the transcription assay but in none of them neither an antagonistic nor agonistic effect was seen. Monomer was tested up to 10:1 in both assays and it had only an agonistic effect. STAT5 assay was repeated three times with the Δ188-190-dimer but otherwise the data is from single experiments.

The obtained luminescence counts of BaF-B03 assay and normalised *Renilla*/firefly luciferase activity in STAT5 assay were divided by the signal obtained without antagonist, hence only rhGH. These B/B0 values were plotted against the molar ratio of antagonist and rhGH. Only B2036 displayed a clear antagonistic effect in both assays. Biological activity of samples with Δ188-190-dimer remained unchanged and that of the monomer samples increased, as could be expected based on the stimulation assays.

4.5 Stability

Two methods were applied for stability studies. Simulating physiological conditions, GH was incubated at 37°C in human serum and its concentration was followed over time. In the second experiment GH was exposed to the digestive enzyme trypsin.

4.5.1 Half-life in serum

Concentrations of wt, phGH and mutant GH were observed over 24 h or 144 h when incubated in low [GH] human serum at 37°C. Samples were collected at different time points and their concentrations were measured by TR-IFMAs. The resulting concentrations were divided by the ones obtained at t(0). Means of these B/B0 values measured by two different antibodies were plotted against the incubation time. Results can be seen in figure 32.

Concentration of wt was not altered during 24 h, whereas concentrations of C182A and C189A dropped rather quickly. Roughly estimated half-lives of C182A and C189A were 4 and 15 h, respectively. C182+9A did not have a reduced stability compared to wt GH. Δ188-190-monomer was clearly less stable than wt GH, but did not drop below 50% during the 24 h incubation. However when it was incubated for 144 h, the concentration dropped more quickly and was below 50% as the 24 h sample was taken. In addition to Δ188-190-monomer, also Δ188-190-dimer, phGH and mutants R77C and D112G were incubated in serum for 144 h. Δ188-190-monomer was the only sample with reduced stability compared to wt and phGH. Δ188-190-dimer had an increased stability instead.

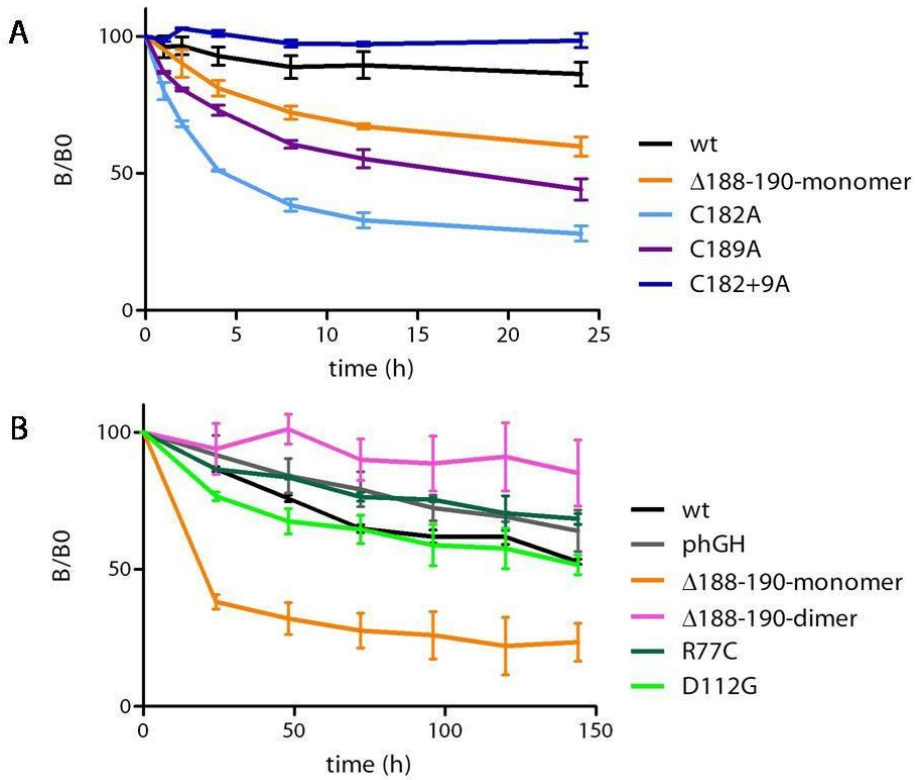

Figure 32: *Half-life in serum.* Wt, Δ188-190-monomer, C182A, C189A and C182+9A were incubated in low [GH] human serum for 24 h (**A**) and wt, phGH, Δ188-190-monomer and -dimer, R77C and D112G for 144 h (**B**). During the 24 h incubation, all mutants except for C182+9A were less stable than wt GH. During the 144 h incubation, Δ188-190-monomer was less stable and Δ188-190-dimer more stable than wt or phGH. R77C and D112G had similar stability as wt and phGH. Bars represent the different concentrations measured by two antibodies.

4.5.2 Trypsin resistance

Resistance of wt and mutant GH against trypsin digestion was studied by incubating the samples with trypsin for 2 h and then observing differences in degradation in Western blot. Results are presented in figure 33.

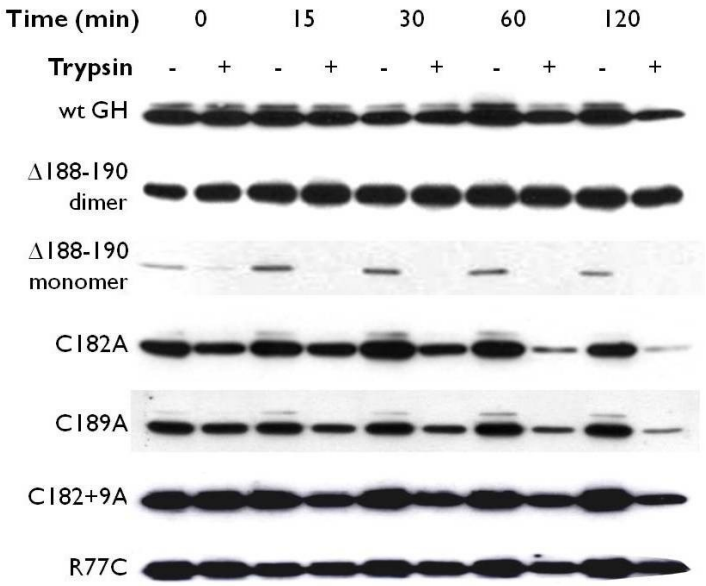

Figure 33: *Trypsin resistance.* Wt and mutant GH were digested with trypsin for 2 h and the degradation was studied by Western blot. Wt GH displayed moderate degradation whereas the mutants C182A and C189A were much more degraded after two hours. Δ188-190-dimer showed very high resistance against trypsin whereas the monomer could not even be detected in the samples incubated with trypsin (except for the 0 min sample). C182+9A and R77C showed degradation comparable to wt.

The results from trypsin digestions were similar to the ones from serum incubations. Wt, R77C and C182+9A were slightly degraded over the 2 h whereas Δ188-190-dimer remained unchanged. C182A and C189A were less stable than wt, and Δ188-190-monomer could barely be detected in Western blot in reducing conditions, as already noticed in chapter 4.2.

5 Discussion

The main objective of this PhD project was to characterize the novel heterozygous GH deletion mutation Δ188-190 found in patients with retarded growth. Along the way two subprojects evolved: 1) investigating the role of the C-terminal disulfide bridge in human GH, which is disrupted in the novel mutant, and 2) further characterizing the previously reported GH mutations R77C and D112G, which also have been identified in heterozygous state in patients with short stature. Mainly the same experimental methods were applied in all projects. The experimental approach is discussed first and then the outcome of each project is discussed in its own chapter.

5.1 An array of methods for characterization of recombinant GH was established

Each mutation was induced to the *GH-1* gene by site-directed mutagenesis after which the cDNA was subcloned into the expression plasmid pcDNA3.1. We used the human cell line HEK-293 for expression of wt and mutant GH. GH was secreted into the growth medium, which was collected for experiments. Western blot analysis showed whether mutants and wt GH were secreted in similar concentrations and their level of dimerization or oligomerization. Exact GH concentrations were determined by TR-IFMAs. The GH-containing supernatants were used as such for the experiments except for the separated monomer and dimer. We tried at first to purify the GH with 10A7- and 5E1-coupled NHS-activated Sepharose 4 Fast Flow resins and beads. With both mAbs the recovery was very low, approximately 10%. With large amounts starting material and severe concentration afterwards one would gain sufficient amounts to be used in the bioassays but since the supernatants turned out to be suitable as such, purification was set aside.

The mutants were characterized in regard to binding affinity, bioactivity and stability. Binding to GHBP was studied by two immunoassays and to GHR by a cell assay. Binding affinities obtained by the different methods correlated well with each other, and if one does not have the possibility to do the cell assay requiring radioactive GH one can quite well rely on the results from the immunoassays. Bioactivity was studied by BaF-B03 cell proliferation assay and STAT5 transcription assay. These two approaches cover different aspects of the bioactivity since one measures signal transduction and the other the growth promoting effect. Cell proliferation was studied in the PRL receptor (PRLR)-expressing Nb2 cells as well but the method was abandoned due to very low reproducibility. Stability was studied by incubation in low GH human serum and by digestion with trypsin.

5.1.1 Secretion

Studying the secretion of a GH mutant and especially its secretion together with wt GH is another important aspect when characterizing GH mutants. All the mutants included in this thesis were found in patients with normal to high GH levels so the secretion did not become a focus of this project. However, secretion and co-secretion with wt GH of the mutants P89L, V110F, I179M and R183H, which were characterized by MSc Maria Hennig, were studied in the mouse pituitary cell line AtT-20. The cells were transfected with either wt GH, wt & mutant GH or mutant GH constructs and GH concentrations in supernatant and cell lysates were measured. One would get a more accurate look at the synthesis and secretion by immunofluorescence staining of GH and the involved cell organelles, Golgi apparatus and endoplasmatic reticulum, as done by Salemi et al. [2005]. In the experiments done in our group it seemed that all the studied mutants were produced in lower amounts than wt GH and especially P89L and V110F even hampered production of wt GH. All in all, the prob-

lem seemed to be in production and not secretion. On the contrary, when Salemi et al. studied P89L and R183H, the problem seemed to be more of the secretory kind [Salemi, 2005].

5.2 The novel GH mutant Δ188-190 has reduced bioactivity but not a high antagonistic potency

5.2.1 Starting point

This project arose in the year 2000 when 10-year-old twin girls were diagnosed with GH deficiency based on low GH and IGF-1 levels. GH treatment was started but the achieved growth acceleration was only modest. The *GH-1* gene of the girls was sequenced and a heterozygous deletion of amino acids 188-190 was discovered. Amino acid 189 is a cysteine forming the C-terminal disulfide bridge of GH and thus such mutation resulted in an unpaired cysteine, C182. The first hypothesis [Weigel, 2004] was that a secretory problem caused the GHD of these patients since this had been observed in patients carrying heterozygous mutations that causes exon 3 skipping [McGuinnes, 2003]. Skipping of exon 3 deletes C53 and thereby leaves C165 unpaired. Disruption of the C-terminal disulfide bridge had not been confronted in a patient before. When a closer look was taken on the serum GH levels of the patients it turned out that there was no secretory problem after all. By using more sensitive immunoassays employing monoclonal antibodies, GH levels *above* the normal range were measured. The GH just could not have been shown in the previous assays since most of it was in a dimeric form. Dimer formation was shown by FPLC analysis, Western blot and TR-IFMAs employing GH dimer -specific monoclonal antibodies. GH dimers and oligomers are also a normal but rather minor component of serum GH [Baumann, 1999].

5.2.2 GHR antagonist hypothesis

High GH levels and low IGF-1 levels, the assumption that also wt GH was present in circulation and the patients' unresponsiveness to GH therapy suggested that the new GH mutant, Δ188-190, could be a GHR antagonist. A similar case had been reported by Takahashi et al. in 1996. A boy had a heterozygous R77C mutation and thus also an unpaired cysteine and it was suggested that this mutant would form a dimer acting as a GHR antagonist. This hypothesis was proven wrong later on: R77C was found to have similar bioactivity as wt GH, and when R77C was combined with wt, the effect was fully synergistic and not antagonistic [Petkovic, 2007(x2)]. Nonetheless, maybe the new Δ188-190 mutant could be a GHR antagonist. Unfortunately, by the time of these findings it was too late to treat the patients with another approach: a combination of somatostatin analogue and recombinant GH. This way the endogenous production of the mutant would have been suppressed and the recombinant GH could have acted unopposed.

Even though it was too late to help the patients carrying the novel mutation – at least until they have children of their own – there was another group of patients that could benefit from this mutant. Namely, a GHR antagonist can be used in treatment of acromegaly. There is an interest to find a new GH antagonist since the only one on the market thus far, Pegvisomant, is extremely expensive and doses as high as 10-20 mg/day are required [Trainer, 2000]. Monthly costs are $2812 on the minimal dose according to the National PBM Drug Monograph for Pegvisomant [Goodman, 2003]. If the deletion mutant dimer would be an effective antagonist as such, no pegylation due to its high stability would be required. Not needing the pegylation would lead to both lower preparation costs and lower doses since the PEG residues make up 48-58% of the molecular weight of Pegvisomant and pegylation leads to a 20-fold decrease in bind-

ing affinity to GHR [Pradhananga, 2002]. Of course, this would require that the antagonistic effect of the Δ188-190 dimer would be at least as high as that of Pegvisomant.

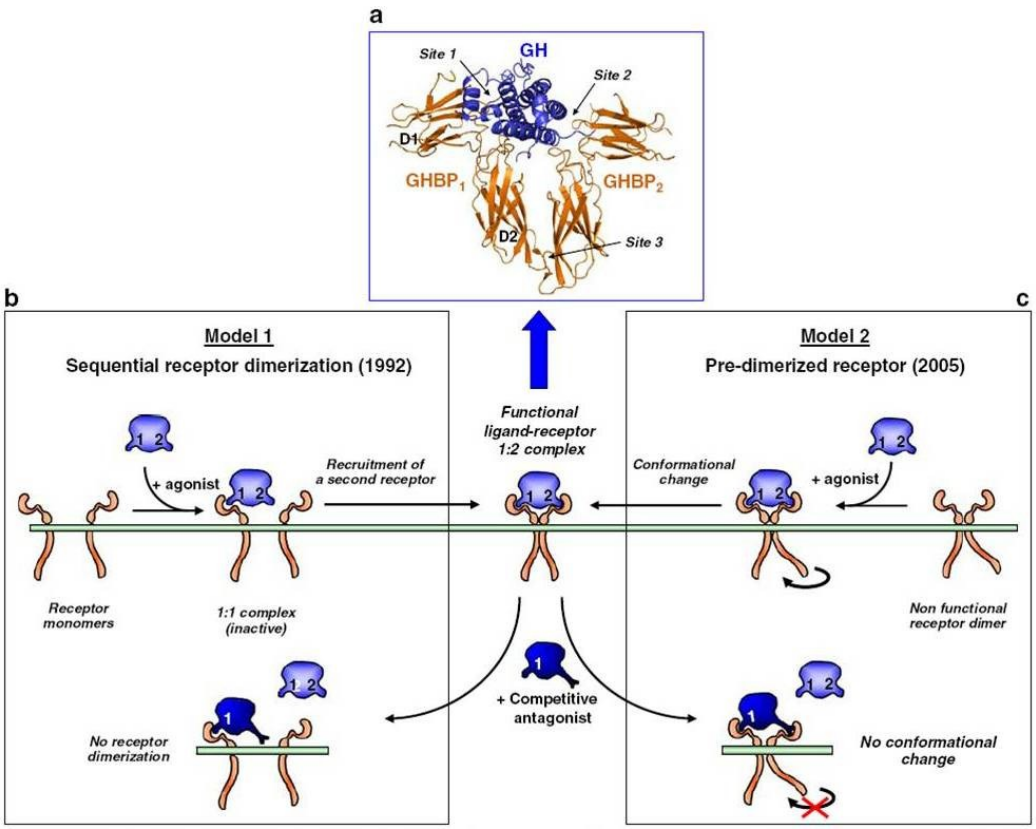

Figure 34: *Antagonist design.* As depicted in model 2 (picture c), the only known GH antagonist, Pegvisomant, binds to GHR by the high-affinity binding site 1 but due to the bulk in binding site 2, the second receptor cannot bind optimally. The conformational change required for signal transduction does not take place and simultaneously the receptor is occupied and wt GH cannot bind. Model 1 (picture b) describes the antagonist hypothesis from the time when Pegvisomant was invented. The figure has been published by Tallet et al. in 2008.

The action of Pegvisomant is based on a G120K mutation. The mutant binds with normal affinity via its high affinity binding site 1 but due to the bulkier lysine instead of glycine at binding site 2, the mutant is not able to induce the conformational change in the receptor required for signal transduction. Mechanism of GH antagonist action is described in figure 34 [Kopchick, 2002; Tallet, 2008]. Since the Δ188-190 mutation is in the proximity of binding site 1 and since disulfide bonds only stabilize the three-

dimensional structure without changing it, it is likely that the intermolecular disulfide bond would be formed between the free cysteines at position 182, leaving only the low-affinity binding site 2s of a mutant dimer unaltered and available for receptor binding. Preferably high-affinity binding would be desired from a GH antagonist.

5.2.3 Experimental approach

As this project was started, only clinical and sequencing data existed for the novel deletion mutant. Serum of the patients had been studied by FPLC, Western blot and TR-IFMAs, indicating that their GH was in dimeric form. We produced the mutant *in vitro* and characterized it regarding its binding affinity to GHR and GHBP, its bioactivity and stability. Antagonistic effect was studied in comparison to B2036, the non-pegylated form of Pegvisomant.

5.2.4 Outcome

5.2.4.1 Different oligomerization pattern *in vitro* and in patient serum, and variation in stabilities

Δ188-190 and wt GH were produced in HEK-293 cells. Because of the patients' high GH levels, impaired secretion was unexpected and since similar amounts of mutant and wt GH were measured in cell supernatant, the secretion was not further investigated. Based on patient data, Δ188-190 was expected to form a dimer but this turned out to be only partly true: in Western blot of the cell supernatants more dimer was detected in Δ188-190 than in wt sample but there was also an approximately equal amount of monomer. Even some trimer was present in the Δ188-190 sample but none in wt. Thus, *in vitro*, the free cysteine drives the Δ188-190 mutants to form covalent disulfide dimers and trimers but monomer is just as abundant, whereas in patient serum trimer was absent and monomer was only present in small amounts, presumably formed by wt GH. One cause is proba-

bly the completely different media that the GH is in: cell culture medium vs. human serum, in which the presumably less stable forms, mutant monomer or trimer, are more prone to breakdown and degradation. The dimer was shown to be extremely stable both in serum incubation test and when digested with trypsin, whereas monomer was very unstable.

Another plausible reason for differences in oligomerization is that the HEK-293 cell culture does not completely resemble the somatotrophs where GH synthesis takes place. For example the oxidative power and presence of protein disulfide isomerases in the ER, where the post-translational modifications take place, could vary between different cell types. In addition, we cannot be sure that the dimer found in patients is a mutant homodimer. Even though it would be energetically favourable to form disulfide bonds between two mutant molecules with free cysteines, the *in vivo* synthesis may lead to formation of a wt/mutant heterodimer or a mixture of all possible homo- and heterodimers. However, the resulting free cysteines would be highly reactive and probably be oxidatively modified, for example by cysteinylation which is common for many plasma proteins [Ghezzi, 2005].

5.2.4.2 Separation of monomer and dimer

In order to study the binding affinity and biological effects of exclusively the mutant homodimer, dimer and monomer in cell supernatant of the Δ188-190 producing cell line were separated by size-exclusion chromatography. The separation was confirmed in Western blot. In reducing conditions the monomeric mutant could barely be detected even though the non-separated Δ188-190 mutant in cell supernatant was detected without problems. This is probably due to partial degradation during the process of production, storage and purification, which differs quite a lot from that of the Δ188-190 produced by transient transfection. First of all, during the production in cell line the medium of the MiniPERM culture was un-

changed for 10 days, after which it was stored at 4°C until chromatography, whereas the transient transfection only required a few days in cell culture conditions, after which the supernatant was stored in -20°C and was ready for analysis as such. Also the chromatography is a harsh process for an unstable molecule like the Δ188-190 monomer.

Since the production of monomeric and dimeric Δ188-190 mutant required vast amounts of starting material and was a rather slow process, one had to be careful deciding which experiments to do with them. Testing the antagonistic effect was our main goal and therefore the focus was on bioassays.

5.2.4.3 Reduced binding affinity to GHR and GHBP

Binding affinity was studied by immunoassays employing GHBP, i.e. the extracellular domain of GHR, and an experiment where the GH would bind to full-length GHR on HEK-293 cell surface. Unlike expected from an antagonist, the mutant displays lower binding affinity than wt GH. In the immunofunctional assay testing the integrity of both GH binding sites only 4% of total Δ188-190 could be measured. In competitive GHBP binding assay the mutant had a significantly lower binding affinity compared to wt GH: approximately 2.8x more mutant was required for same effect (IC50). When binding to GHR, 2.5x more mutant was needed for same effect. These assays were not done with the separated monomer and dimer, except for the first step of competitive GHBP assay where concentration at 10% maximal binding was determined. However, the 10% of maximum could not be reached with the dimer at all. Approximately 2.5x the amount of wt was needed to achieve the same binding with monomer, and theoretically, one would need 200x the amount of wt to achieve the same binding with the dimer. Thus the binding affinity of the Δ188-190 mutant – especially in its homodimeric form – does not speak for the antagonist hypothesis.

5.2.4.4 Reduced ability to activate the Jak/STAT pathway and to induce cell proliferation

Δ188-190 in cell supernatant is biologically less active than wt GH. In BaF-B03 cell proliferation assay the EC50 of mutant is approximately twice as high as that of wt. Mutant-induced activation of STAT5 dependent transcription was lower than wt-induced activation at each concentration. Both assays were also done with the separated monomer and dimer, both of which were even less bioactive than the mutant in cell supernatant. The dimer was not able to induce STAT5 dependent transcription at all. However in the proliferation assay it was active at high concentrations. One possibility is that the dimer is broken and the effect is in fact induced by monomer but results from stability tests do not support this - if a 7-day incubation in human serum does not break the dimer, a 24 h incubation in cell culture is unlikely to do it.

The results obtained with separated monomer and dimer do not correlate with the results obtained with the mutant-containing cell supernatant. Based on Western blot, the supernatant contains approximately equal amounts of dimer and monomer and therefore one would expect that its binding affinity and biological activity would be close to the mean of those of monomer and dimer. Instead, the binding affinity of Δ188-190 in supernatant resembles that of monomer and in the bioassays monomer is even less bioactive than the supernatant sample. This is not due to the use of wt GH and rhGH because they have similar activities in both the proliferation assay and the transcription assay. As already mentioned the monomer cannot be recognized in Western blot and is probably partly degraded during the lengthy production. This could also lead to the rather low bioactivity and binding affinity of the monomer but cannot explain the relatively high bioactivity and binding affinity of the Δ188-190 supernatant.

5.2.4.5 Inhibition of wt action

A 10-20 mg daily dose of Pegvisomant results in 10,000-100,000 ng/ml serum concentration. GH levels in acromegalic patients are usually around 10 ng/ml [Trainer, 2000], and thus a 1,000-10,000:1 Pegvisomant:serum GH mass-to-mass ratio is needed in order to adequately reduce serum IGF-1 levels of the patients. Since the PEG5000 residues make up approximately half of the mass of Pegvisomant and since the non-pegylated form of Pegvisomant, B2036, has a 30-40-fold higher affinity to GHR [Ross, 2001], one could estimate that a 10-200:1 B2036:GH ratio suffices for an antagonistic effect. B2036 turned out to be effective already at a 2:1 antagonist:rhGH ratio in the STAT5 transcription assay and at 50:1 in the proliferation assay. The latter proportion would maybe have been lower if a lower concentration of rhGH would have been applied. With B2036 this was not a problem since high concentrations were available but by applying a lower rhGH concentration, a higher Δ188-190:rhGH proportion could have been achieved.

Inhibition was studied with the separated Δ188-190 monomer and dimer. Monomer had only an agonistic effect whereas dimer had no effect at all. Monomer was tested up to 10:1 ratio in both assays and dimer up to 10:1 in STAT5 assay and 5:1 in BaF-B03 assay. One has to keep in mind that the molar ratio of dimer is just half the mass-to-mass ratio. In the patients the molar dimer to monomer ratio was 3-5:1. Hence we expected that the possible antagonistic effect of the Δ188-190 dimer would be prominent at a lower mutant:wt ratio than that of Pegvisomant or B2036. Unfortunately in the proportions that we studied no antagonistic effect of the dimeric Δ188-190 mutant could be observed.

5.2.5 Conclusions

Even though the binding affinity of the Δ188-190 mutant is low and it does not seem to inhibit the action of wt GH *in vitro*, it cannot be ruled out that it would be an antagonist. Since the dimer is extremely stable, it is the most abundant GH isoform in the circulation. Even with its reduced binding affinity, it confronts a GHR much more often than the monomer and thus could interfere with binding of the monomer or rhGH given as therapy. But even if the dimer has an antagonistic effect, it cannot be a very potent antagonist: the patients were not completely unresponsive to GH therapy and the low binding affinity to GHR will be a problem when a high-potent antagonist is being developed.

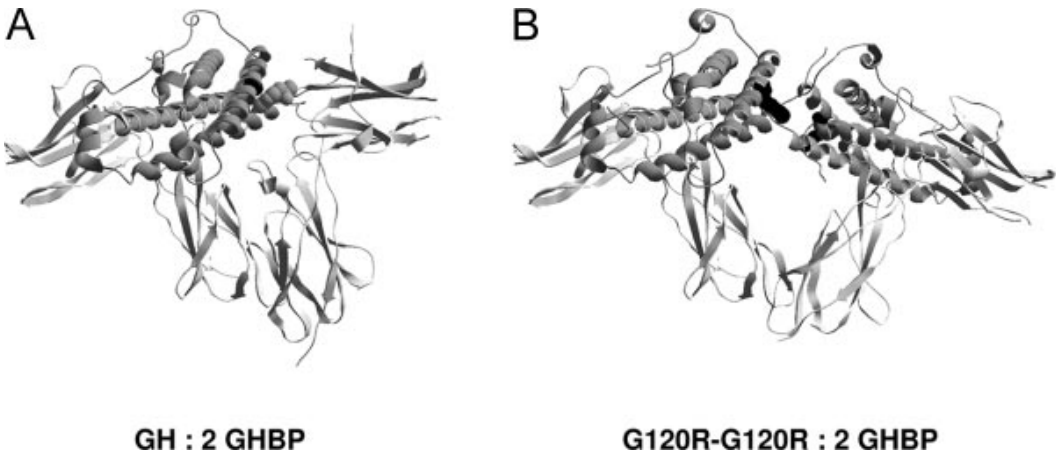

Figure 35: *GHR can be activated by a GH dimer.* In figure A is the common model of single GH molecule binding two GHBP molecules. In figure B is a dimer of the GHR antagonist G120R bound to two GHBP molecules. According to Yang et al., this dimer is able to activate the receptor even though its monomeric form is not. The figure was published by Yang et al. in 2008.

Yang et al. [2008] studied two artificial GH dimers. The wt-wt dimer had all four binding sites available and the G120R-G120R had only two binding site 1s since the binding site 2s were located in the interface. G120R-G120R bound by two GHBP molecules is presented in figure 35. The mutant G120R is the GHR antagonist that lead to development of Pegvisomant, where the mutation was changed to G120K [Chen, 1990; Kopchick,

2002]. It was shown that both a wt-wt dimer and a G120R-G120R dimer were able to activate GHR. This suggests that the GHR dimer is much more flexible than generally believed. Therefore, the Δ188-190 dimer should not be too large for GHR binding either. However as already mentioned, since the mutation is adjacent to binding site 1, the dimer is probably built in such way that binding site 1 becomes hidden and the dimer has two low affinity binding site 2s exposed. From this point of view the low binding affinity is expected.

Our momentary hypothesis is that the reason for the retarded growth in patients carrying the heterozygous Δ188-190 mutation is that the mutant forms a dimer which is able to hamper action of wt GH. Very high stability of the dimer leads to its accumulation and therefore, despite of low receptor binding affinity, it has the quantitative advantage against the wt GH. However, due to some contradictory data, final conclusions cannot be made yet. For example, it cannot be ruled out that the GH dimer found in the patients would be a wt/Δ188-190 heterodimer or a mixture of hetero- and homodimers. This will be further discussed in chapter 6.2.

5.3 The C-terminal disulfide bridge is crucial for full functionality of GH binding site I

5.3.1 Starting point

An important feature of the novel Δ188-190 mutation was that it caused disruption of the extremely conserved (see table 1) C-terminal disulfide bridge of GH between C182 and C189. Its role has been investigated only scarcely and mostly decades ago. Based on experiments with bovine GH, this disulfide bridge has been claimed to be unimportant for secretion, receptor binding and growth promoting effect [Chen, 1992]. However its conservation and location within GH binding site 1 [Cunningham, 1989 & 1991] speak for its importance. The disulfide bridge between C53 and C165

has been more under interest than the C-terminal one, partly because it is disrupted in patients with a heterozygous Δ32-71 mutation (Δexon3), having a dominant negative effect. In addition a homozygous C53S mutation has been identified in a boy with retarded growth. Unlike the Δ32-71 mutation, this one did not show a dominant negative effect [Besson, 2005].

Cunningham et al. [1989, 1991] performed several alanine mutations in order to find out which amino acids are important for receptor interaction. They showed that the cysteine C182 is involved in binding site 1 and that the mutant C182A displays reduced receptor binding. They could not analyse mutant C189A because of its low expression in *E. coli* [Cunningham, 1989]. In order to gain more information about the role of the C-terminal disulfide bridge, we converted one or both of the participating cysteines to alanines, creating mutants C182A, C189A and the double mutant C182+9A. These mutants were characterized similarly as Δ188-190.

5.3.2 Outcome

5.3.2.1 Normal secretion but low stability caused by an unpaired cysteine

In the beginning only mutants C182A and C189A were constructed and produced. In our HEK-293 cells even C189A was secreted in similar amounts as wt GH. A special feature of C182A and C189A was very low stability in comparison to wt GH. This made us speculate that the free cysteine and not the structural role played by the disulfide bridge could be responsible for the rapid degradation, both when incubated in human serum and digested with trypsin. In order to investigate this, a third mutant, where both involved cysteines were converted to alanines, C182+9A, was constructed and characterized. It turned out that, in fact, the stability was recovered by removing the free cysteine. Thus the disulfide bridge only contributes to stability of the molecule by keeping the reactive cysteines

from reacting with other surrounding molecules. It is actually all in all more accurate to investigate the role of a disulfide bridge by removing both involved cysteines. Betz [1993] studied the meaning of disulfide bonds for protein stability in general but concluded that it is hard if not impossible to study their role by removing or mutating a cysteine since one cannot maintain the exact polarity and volume of the disulfide state. In addition, the unpaired cysteine of a protein-S-H can be oxidised forming a protein-S-X, X being an available molecule in the expression system, such as glutathione or free cysteine [Ghezzi, 2005].

5.3.2.2 Integrity of the C-terminal disulfide bridge critical for binding affinity to GHR and GHBP

As demonstrated in figure 36A, the amino acids of the C-terminal disulfide bridge of GH are within binding site 1 in direct contact to the extracellular domain of GHR (GHBP). Therefore it was expected that it has a high importance for binding affinity. All the mutants with disrupted C-terminal disulfide bridge had a significantly reduced binding affinity to GHBP and to full-length GHR and it did not make a difference whether a free cysteine was present (binding to full-length GHR was not analysed with C182+9A). Consequently, it is probable that the free cysteines did not react with any large molecules or perhaps the cell culture conditions were not very oxidative and the cysteines remained in the thiol (protein-S-H) state.

C189A was the disulfide bridge mutant with lowest binding affinity in all binding experiments, thus a free cysteine at position 182 or the alanine at 189 hampers the binding more than vice versa. Conservation of the disulfide bridge in GH family members and also in different species (see table 1) is probably due to its location within the binding site. In figure 36B the GH family member prolactin is bound to the ECD of its receptor and even though the second cysteine (C199) cannot be seen, it is apparent that the disulfide bridge is in close contact to the receptor.

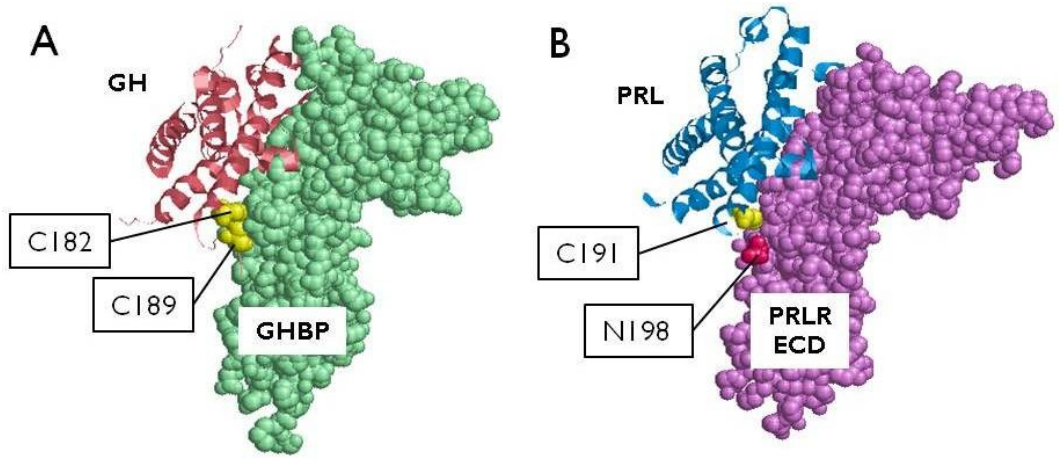

Figure 36: *The C-terminal disulfide bridge is located within binding site 1.* **A.** A pink GH molecule is bound to a light green GHR ECD (i.e. a GHBP). C182 and C189 of GH are yellow and both are in contact with the receptor. **B.** A light blue PRL molecule is bound to a violet PRLR ECD. C-terminal disulfide bridge of PRL is formed between C191 (yellow) and C199 but the latter is not included in the 3D model. The last amino acid included is N198, which is coloured magenta. GH-GHBP model is by Clackson et al. [1998] and PRL-PRLR ECD model by Svensson et al. [2008].

5.3.2.3 Bioactivity close to wt despite of lower binding affinity

In addition to the lowest binding affinity, C189A turned out to have the lowest bioactivity of these mutants. Even though C182A and C182+9A had a reduced binding affinity as well, their bioactivity was similar to wt GH, except for C182A at the highest concentration in STAT5 assay. C189A on the other hand displayed a lower bioactivity than wt in both BaF-B03 proliferation assay and STAT5 assay. C189A is structurally more similar to Δ188-190 than C182A or C182+9A, and – based on our data – also functionally.

5.3.3 Conclusions

C182A and C189A had very low stability in comparison to wt GH. Studying the mutant C182+9A, which does not have the C-terminal disulfide bridge but does not have a free cysteine either, revealed that the reduced stability had been caused by the free cysteine of C182A and C189A and not by the absence of the disulfide bridge per se. However, as was seen in case of R77C (discussed in chapter 5.4), stability of a GH molecule is not always

reduced if a free cysteine is present. This could be due to the different locations of the free cysteines even though all the studied mutants with a free cysteine, i.e. Δ188-190, C182A, C189A and R77C, dimerize more than the ones with a paired number of cysteines, suggesting that even the free cysteine of R77C is exposed and reactive. Evolutionally the contribution of the C-terminal disulfide bridge to stability may still be important since the deletion of one cysteine is more likely to happen than removal of both of them. In addition, reduced binding to GHBP reduces the stability even more due to loss of the protective effect of GHBP.

The importance of the C-terminal disulfide bridge for binding affinity did not come as a surprise because of its location within GH binding site 1. Despite of this, the mutants were able to induce signal transduction and cell proliferation almost as wt GH, only bioactivity of C189A was somewhat lower. It is not unusual that binding affinity does not correlate with bioactivity [Haugh, 2004]. Figure 37 describes a kinetic model, where all the different destinations of a GH-GHR complex are taken into account. Unfortunately this model was created before the current idea of a preexisting receptor dimer was more widely accepted. However, the most important point is that receptor binding is an extremely dynamic process and one has to take into account at least GHR synthesis, internalization of the GH-GHR complex and its recycling and degradation.

According to Haugh [2004], an internalized 1:2 GH-GHR complex can only be degraded but a 1:1 complex can also be recycled. It would be interesting to know what would happen with a 1:2 complex if the GH was a mutant with low binding affinity and reduced ability to initiate signal transduction. If this would turn on the recycling system, we would have an explanation for the normal bioactivity despite of poor binding affinity.

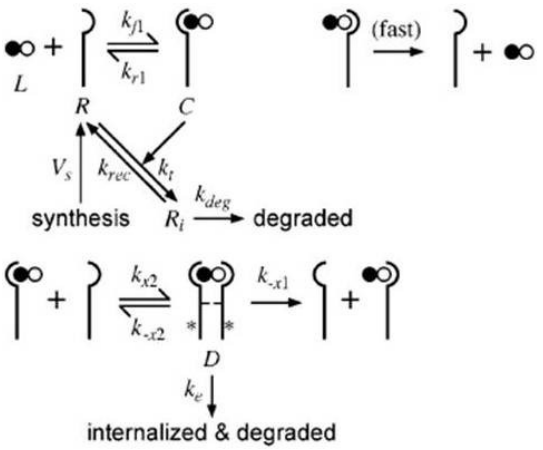

Figure 37: *Illustration of GH receptor binding and trafficking by a kinetic model [Haugh, 2004].* Unfortunately the model is not completely accurate because of the outdated pattern of receptor binding but it gives an idea of the complexity of the process. L is an extracellular ligand, black and white circles representing GH binding site 1and 2, respectively. R is a free GHR, C is a 1:1 GH-GHR complex (inaccurate) and D is a 1:2 GH-GHR complex.

5.4 GH mutants R77C & D112G present in patients with short stature barely differ from wt GH *in vitro*

5.4.1 Starting point

Both R77C and D112G were first identified by Takahashi et al. [1996 & 1997]. R77C drew some attention in the medical circles since it was claimed to form a dimer with an antagonistic effect [Chihara, 1998; Takahashi, 1996]. Ten years later, however, the same mutation was found in another family by Petkovic et al. [2007(x2)], and they showed that R77C did not have any antagonistic potency. Since we were studying a possible antagonist as well, it was interesting to take this mutant along to our studies. D112G was included since no new data about it had been published since the first publication by Takahashi et al. in 1997, where it was shown to have a reduced binding affinity and bioactivity. In fact, all the missense mutants shown in figure 12 were cloned and studied to some extent, except for the recently published D116E [Dateki, 2009]. Results from the characterization of P89L, V110F, I179M and R183H can be found in the Master's thesis of Maria Hennig [2010].

5.4.2 Slight reduction in bioactivity the only difference to wt GH

Both R77C and D112G behaved very similarly as wt in all our experiments, except for the STAT5 transcription assay, where both had a slightly reduced activity in comparison to wt GH. Binding affinity of R77C was only tested to GHBP but since in all other cases the two binding affinities correlated perfectly, we assume that R77C does not have a reduced binding affinity to GHR either. R77 was not included in Cunningham's epitope mapping since it was not part of an epitope that was believed to take part in receptor binding. D112A was reported to have the same receptor dimerizing activity as wt GH [Cunningham, 1989 & 1991]. On the GHBP-GH-GHBP 3D model (see picture 38) it seems that R77 indeed is far from both binding sites but D112 is quite close to binding site 2.

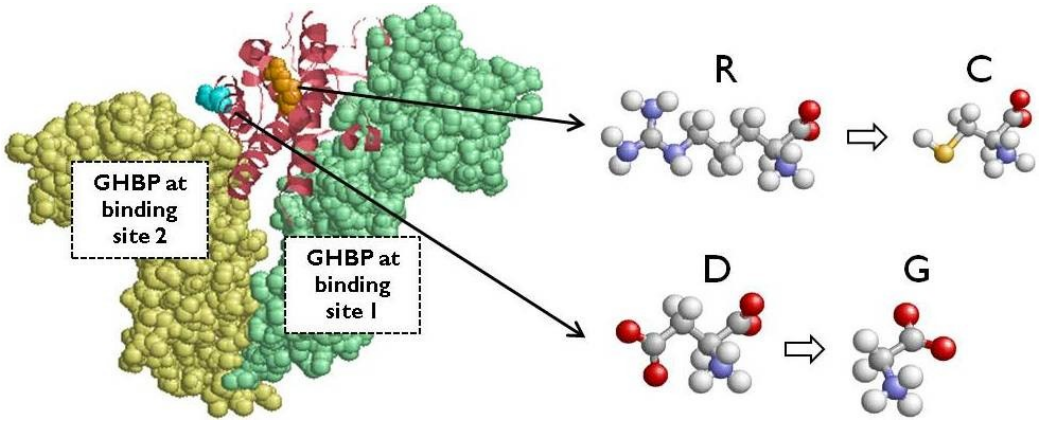

Figure 38: *Affect of mutations R77C and D112G on GH-GHBP interaction.* On this model of a 1:2 GH:GHBP complex [Sundström, 1996] one can see that the R77C (orange) mutation is unlikely to affect receptor binding. D112 (cyan) is rather close to binding site 2 but since it is substituted with a tiny glycine, it is improbable to hamper binding either. Amino acids' structures are from PDB. Arginine, cysteine and aspartic acid are hydrophilic and glycine is amphipathic, thus the polarities do not cause problems either. Structures of all amino acids can be found in appendix I.

In the patient D112 is substituted with a glycine which is much smaller than the original aspartic acid and therefore is not likely to interfere with site 2 binding. None of the substitutions, R77C or D112G, change polarities either and thus would not be expected to affect protein folding. Takahashi et al. [1997] showed that D112G tended to form a 1:1 complex with GHBP

rather than 1:2 complex formed by wt GH. Yet, the fact that D112G had a very similar IC50 as wt GH in binding to the full-length GHR indicates an unaltered binding affinity.

As already mentioned in chapter 5.3.3, the free cysteine in R77C causes it to dimerize somewhat more than wt but does not seem to affect any other properties of the molecule. It is surprising that a free cysteine at C-terminus remarkably reduces the molecule's stability as seen in case of C182A and C189A, but the C77 on helix 2 has no consequence in this regard. An explanation could be that the C77 is deeper embedded within the protein and therefore less reactive than the C-terminal cysteines.

5.4.3 Conclusions

Our results were not consistent with the ones published by Takahashi et al. [Chihara, 1998; Takahashi, 1996 & 1997]. Data concerning R77C was already disproved by Petkovic et al. [2007(x2)] and our results are very similar to theirs – R77C resembles wt GH both in receptor binding and bioactivity although in our STAT5 assay the bioactivity was somewhat reduced. The explanation offered by Petkovic et al. is that the mutant activates GHR/GHBP transcription less than wt GH and therefore also an effect on somatic growth could be seen. This approach was not included in our studies. We would still like to speculate that the slight reduction in bioactivity could play a role in the patients. It is known that one functional allele of the *GH-1* gene is sufficient for normal growth, as found in heterozygous relatives of type IA IGHD patients [Binder, 2002; Mullis, 2005 & 2007]. Homozygosity on the other hand leads to severely retarded growth. Situation of a patient with heterozygous expression of bioinactive GH is quite different from the heterozygous deletion. The GH mutant with reduced bioactivity still occupies the receptor and only half of the GH in circulation is fully active.

In case of R77C, relatives also carrying the mutation did not have retarded growth. Height of the Japanese boy was -6.1 SD at the age of 5 years but that of his father, who also carried the mutation, was only -0.2 SD. However, presence of the mutant could not be detected by isoelectric focusing in the father's serum. Approximately twice as much wt GH as mutant was found in the boy. Genomic imprinting could explain the different expression patterns but was not further investigated [Chihara, 1998; Takahashi, 1996]. The Syrian boy with the same mutation had a height of -2.5 SD at the age of 6 but finally reached -1.9 SD without GH therapy. The boy's mother, aunt and grandfather, who also carried the mutation, had heights of -1.4, -1.3 and -1.7 SD, whereas heights of non-affected family members were -0.8 to -1.5 SD. Mutant was present in similar concentration as wt GH in the boy and the mother, as measured by isoelectric focusing [Petkovic, 2007(x2)].

D112G was only found in one girl, in almost four times higher amount than wt GH. Her height was -3.6 SD at the age of three but a notable improvement was achieved with GH therapy. According to Takahashi et al. [1997] the mutant was biologically inactive and had reduced ability to form a 1:2 complex with GHBP. All we found was a slight reduction in ability to induce Jak/STAT signalling but, as mentioned above, maybe such a reduction is enough to cause the phenotype in a patient. The much higher concentration of mutant in comparison to wt in the patient could not be explained by different stabilities and Takahashi et al. [1997] did not have an explanation for this either. It is however questionable if isoelectric focusing of one serum sample is enough to draw conclusions of an overall expression pattern.

The poor correlation of patient phenotypes and *in vitro* results are not explicable by alterations in genes involved in the downstream effects of GH. For instance, the several involved transcription factors are not GH specific

and thus impairment on that level would result in a more complex and severe phenotype. Defects in IGF-1, unlike GH, would lead to severe pre-natal growth retardation [Mullis, 2005].

6 Outlook

6.1 Future application of the established methods

We now have established an array of methods for a fairly rapid characterization of a GH mutant or for instance a GH variant. There are some pitfalls that are important to keep in mind when applying the methods.

Determination of concentration is an extremely important step and mistakes can distort all results. All experiments are based on dose-response effects and thus an inaccurate concentration directly leads to a false outcome. GHBP binding assay is an exception since a concentration with a signal corresponding to that of wt is determined. We tried to find the most suitable monoclonal antibodies for the TR-IFMAs by taking their binding epitopes into account and finally through trial and error. One can predict quite well from the location of the mutation which antibodies not to use. A more accurate way to determine protein concentration is by measuring the OD. However, even after purification by affinity chromatography the sample contained at least light and heavy chains of the used antibody, which would interfere with the measurement.

Choice of standard is important for determination of concentration. Traditionally the phGH preparation 80/505 has been thought to best resemble the real situation – a mixture of isoforms and oligomers. However the current reference standard is the synthetic 88/624 because of its high purity and reproducibility [Jansson, 1997]. We used the phGH 80/505 always when determining concentration by TR-IFMAs and 88/624 for the immunofunctional assay and for cell experiments with Δ188-190 monomer and dimer. The different concentration of wt GH in TR-IFMA and immunofunctional assay is probably due to the different standards.

The experiments of this project were done over more than a two years time and it turned out that variation can be great between assays, especially if they were done with long intervals between experiments. It became crucial that a mutant was always compared to wt that was analysed in the same assay. The two GHBP binding assays for C182 and C189A, done with different stocks of unlabelled GHBP (see fig. 25), are a good example of variation between assays. Similarly, different stocks of GHBP-b caused the maximal signal to vary from 968,000 to 1,640,000 counts (see fig. 24).

One has to keep in mind that *in vitro* characterization of a human GH mutant is only an initial step in analysing its mechanism of action. The path starting from the production of the mutants in non-somatotrophs to their characterization in closed systems is full of divergences from the human system. And as was seen in the case of R77C, even the human system can react in various ways to the same mutation.

6.2 Ruling out wt/Δ188-190 heterodimer

There are still some open questions regarding the Δ188-190 GH mutant. It would be important to verify that the dimer found in patients is actually a mutant/mutant homodimer as hypothesised and not a wt/mutant heterodimer or a mixture of the two. This could be done by mass spectrometry (MS), based on the different trypsin digestion patterns of wt and Δ188-190 mutant. First it has to be made sure that the three amino acid difference in the C-terminal fragment seen in figure 35 actually can be seen in the spectrum. Kohler et al. [2009] have successfully identified different growth hormone isoforms by a similar method but the 0.27 kD difference of our molecules is rather small in comparison to the 2 kD difference between 20 and 22 kD GH, for instance. Even if the difference can be detected, we do not unfortunately have enough patient serum in order to analyse the dimer of the patients. We would have to settle for analysing

the dimer formed after co-transfection with wt and Δ188-190 constructs. The rat somatotroph cell line GH3 would optimally be used for this purpose but their challenging cultivation and rather low yield might be a problem. The mouse pituitary cell line AtT-20 would probably be the second best choice. Dimerization or oligomerization of GH in different cell lines was not studied during the PhD project but would be something worth taking a look at – preferably before large-scale co-transfections.

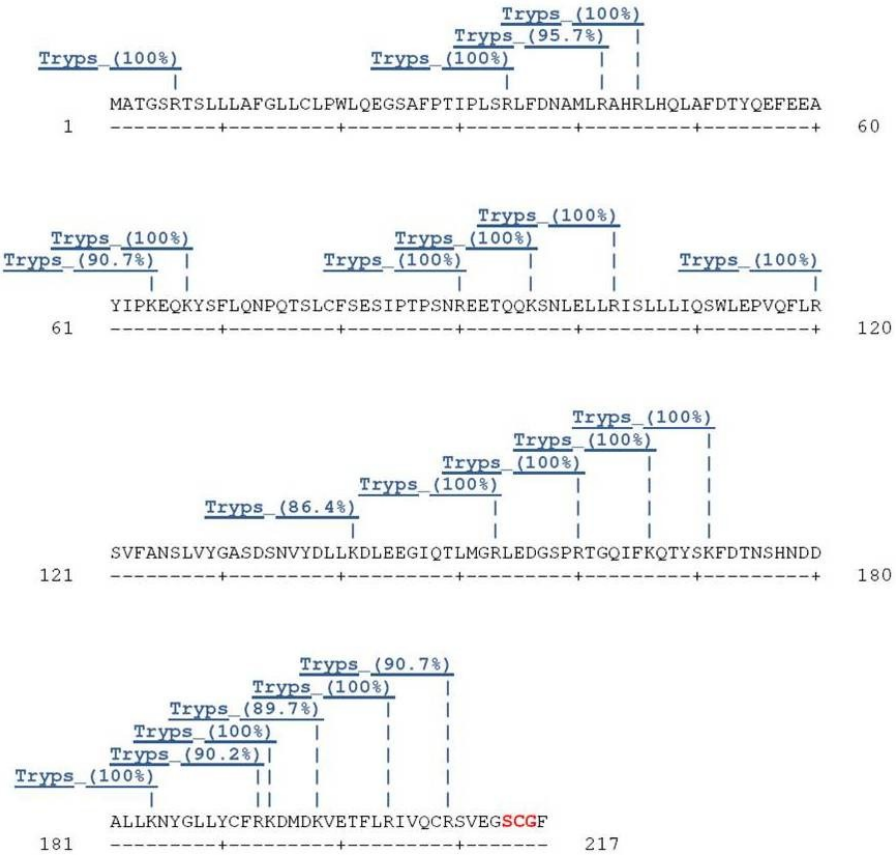

Figure 39: *Trypsin digestion map of hGH (including signal sequence).* Amino acids 188-190 are coloured red. The percentage after each trypsin cleavage site is the probability of trypsin digestion. The map was created with PeptideCutter, a service provided by SwissProt.

Prior to analysis, the background has to be reduced and therefore the GH has to be purified using mAb 10A7-coupled beads, despite of the unsatisfactory yield (see chapter 5.1). The result is separated on SDS-PAGE and the gel is stained by a MS-compatible staining method, such as Coomassie

staining. The desired band is then cut out, trypsinised and analysed by matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) MS. Dr. Katharina Janek from the Institute of Biochemistry at Charité Universitätsmedizin has kindly promised to do the analysis after that our protein is visible on the gel. First wt and Δ188-190 will be analysed in parallel and, if a difference in the spectrums can be seen, the dimer band from a co-transfection will be analysed. If the mutant clearly predominates, we can assume that formation of a mutant homodimer is preferred over a wt/Δ188-190 heterodimer.

6.3 *In vivo* experiments

An *in vitro* characterization can only give directional data about the investigated molecule. The subsequent step would be to study the mutants in an animal model. We are collaborating with Professor John Kopchick at Ohio University in order to create a mouse model expressing the Δ188-190 mutant. He played an important role in development of Pegvisomant and has ample experience in GH mouse models [Chen, 1990; Kopchick, 2003]. All he needs from us are plasmid constructs containing the wt and mutated *GH-1* gene, which then will be subcloned into a suitable vector for microinjection into male pronuclei of fertilized C57BL56/SJL mouse eggs [Chen, 1990; McGrane, 1988]. Once positive transgenic mice expressing either wt or mutant hGH are born, their growth will be observed.

Another approach for *in vivo* studies is to inject the mutant into hypophysectomised rats as done in recent studies concerning a GH ligand-receptor fusion molecule [Ferrandis, 2010; Wilkinson, 2007]. A large amount of the Δ188-190 mutant will be needed and is currently being produced using the stably transfected HEK-293 cell line. Dimer will again be separated from monomer as described in chapter 3.3.2. It is questionable if the resulting dimer is pure enough for the experiment as such. Wilkinson et al. purified the examined fusion protein by affinity purification employing a mono-

clonal anti-GH antibody, similarly as we tried to purify the mutants (see chapter 5.1).

6.4 Looking for antagonists

Since the GH mutant Δ188-190 did not appear to be a very potent antagonist, we started the construction of a series of cysteine mutants, hoping that they would form covalent dimers with antagonistic potential. Yang et al. showed that both a wt-wt and a G120R-G120R dimer (see fig. 35) were able to induce signal transduction, albeit to a lesser extent than wt monomer [Yang, 2008]. This encourages the idea that a GH dimer actually can bind to a dimerized GHR. We will only need to find the optimal disulfide bridge location for maximal binding not leading to signal transduction. Suitable locations for mutations were estimated by *in silico* modelling using RasMol.

6.5 Epilogue

If new *GH-1* mutations related to short stature are to be identified, their mechanism of action can at least roughly be determined by the array of experiments that we established. Fast characterization could help to treat the patients, especially if they do not respond to GH therapy.

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Appendices

I. Amino acids [Voet, 2004]

	Amino acid	Abbr.	Structure formula
nonpolar	Glycine	G/Gly	NH_2-CH_2-COOH
	Alanine	A/Ala	H_3C-CH(NH_2)-COOH
	Isoleucine	I/Ile	H_3C-CH_2-CH(CH_3)-CH(NH_2)-COOH
	Leucine	L/Leu	$(H_3C)_2$-CH-CH_2-CH(NH_2)-COOH
	Methionine	M/Met	H_3C-S-$(CH_2)_2$-CH(NH_2)-COOH
	Phenylalanine	F/Phe	Ph-CH_2-CH(NH_2)-COOH
	Proline	P/Pro	NH-$(CH_2)_3$-CH-COOH |_____|
	Tryptophan	W/Trp	Ph-NH-CH=C-CH_2-CH(NH_2)-COOH |_____|
	Valine	V/Val	$(H_3C)_2$-CH-CH(NH_2)-COOH
polar uncharged	Asparagine	N/Asn	H_2N-CO-CH_2-CH(NH_2)-COOH
	Cysteine	C/Cys	HS-CH_2-CH(NH_2)-COOH
	Glutamine	Q/Gln	H_2N-CO-$(CH_2)_2$-CH(NH_2)-COOH
	Serine	S/Ser	HO-CH_2-CH(NH_2)-COOH
	Threonine	T/Thr	H_3C-CH(OH)-CH(NH_2)-COOH
	Tyrosine	Y/Tyr	HO-Ph-CH2-CH(NH_2)-COOH
polar charged	Arginine	R/Arg	HN=(NH_2)-NH-$(CH_2)_3$-CH(NH_2)-COOH
	Aspartic acid	D/Asp	HOOC-CH_2-CH(NH_2)-COOH
	Glutamic acid	E/Glu	HOOC-$(CH_2)_2$-CH(NH_2)-COOH
	Histidine	H/His	NH-CH=N-CH=C-CH_2-CH(NH_2)-COOH |_____|
	Lysine	K/Lys	H_2N-(CH2)$_4$-CH(NH_2)-COOH

II. Genetic code [Voet, 2004]

<table>
<tr><th>First position (5′ end)</th><th colspan="4">Second position</th><th>Third position (3′ end)</th></tr>
<tr><td></td><td>U</td><td>C</td><td>A</td><td>G</td><td></td></tr>
<tr><td rowspan="4">U</td><td rowspan="2">Phe</td><td rowspan="3">Ser</td><td rowspan="2">Tyr</td><td rowspan="2">Cys</td><td>U</td></tr>
<tr><td>C</td></tr>
<tr><td rowspan="3">Leu</td><td rowspan="2">STOP</td><td>STOP</td><td>A</td></tr>
<tr><td>Trp</td><td>G</td></tr>
<tr><td rowspan="4">C</td><td rowspan="4">Pro</td><td>His</td><td rowspan="4">Arg</td><td>U</td></tr>
<tr><td>C</td></tr>
<tr><td rowspan="2">Gln</td><td>A</td></tr>
<tr><td>G</td></tr>
<tr><td rowspan="4">A</td><td rowspan="3">Ile</td><td rowspan="4">Thr</td><td rowspan="2">Asn</td><td rowspan="2">Ser</td><td>U</td></tr>
<tr><td>C</td></tr>
<tr><td rowspan="2">Lys</td><td rowspan="2">Arg</td><td>A</td></tr>
<tr><td>Met</td><td>G</td></tr>
<tr><td rowspan="4">G</td><td rowspan="4">Val</td><td rowspan="4">Ala</td><td rowspan="2">Asp</td><td rowspan="4">Gly</td><td>U</td></tr>
<tr><td>C</td></tr>
<tr><td rowspan="2">Glu</td><td>A</td></tr>
<tr><td>G</td></tr>
</table>

III. Suppliers of chemicals and reagents, laboratory equipment, kits, consumables and software

Chemical, reagent	Supplier
100 bp DNA ladder	Invitrogen
1 kb DNA ladder	Invitrogen
^{125}I-hGH	MP Biomedicals
Acrylamide/Bis Solution, 30%	Biorad
Agarose	Sigma
Amersham ECL™ Western Blotting Detection Reagents	GE Healthcare
Amersham Hyperfilm	GE Healthcare
Ampicillin	Invitrogen
APS	Biorad
Biowhittaker® Pro293a-CDM medium	Lonza
Boric acid	Sigma
Bovine γ-globulin	Sigma
Bromophenol blue	Sigma
BSA	Sigma
$CaCl_2$	Sigma
CYBR® Safe DNA gel stain	Invitrogen
ddH_2O	VWR
Dexamethasone	Sigma
DMEM	Invitrogen
DMEM:F12, 1:1	Invitrogen
dNTP mix	Invitrogen
DTPA	Sigma
EDTA, 0.5 M	Sigma
EtOH	Merck
FCS	Thermo Scientific
Geneticin (G418)	Invitrogen
Gentamicin, 1 mg/ml	Sigma
Glucose	Sigma
Glycerol	Sigma
Glycine	Merck

HAc	Merck
HCl, 25%	Merck
HEPES	Invitrogen
HRP-labelled α-mouse antibody	GE Healthcare
HS	Invitrogen
Isopropanol ultra for molecular biology	Sigma
Kaleidoscope Prestained Protein Standard	Biorad
KCl	Sigma
KH_2PO_4	Sigma
KHP	Sigma
Lane Marker Sample Buffer	Thermo Scientific
LB Agar tablets	Sigma
LB Broth tablets	Sigma
L-glutamine, 200 mM	Invitrogen
Lipofectamine™ 2000	Invitrogen
Low DNA Mass™ Ladder	Invitrogen
MeOH	Merck
Na_2HPO_4	Sigma
$Na_2HPO_4x12H_2O$	Sigma
NaN_3	Sigma
NaCl	Merck
NaOH, 2 M	Merck
Opti-MEM® Reduced Serum Medium	Invitrogen
PBS tablets	Invitrogen
Penicillin-Streptomycin, 10,000 U/ml; 10,000 µg/ml	Invitrogen
***Pfu* DNA polymerase, 0.3 U/µl**	Promega
***Pfu* reaction buffer, 10x**	Promega
phGH, IRP 80/505	NIBSC
Primers	Invitrogen
Pyruvate, 100 mM	Invitrogen
Restriction enzymes, 10 units/µl, and corresponding REact® buffers	Invitrogen
rhGH, IRP 88/624	NIBSC
RPMI-1640	Invitrogen

Salmon sperm	Invitrogen
SDS	Biorad
Sheep serum	Sigma
Skim milk	Merck, Sigma
Streptavidin-Eu	PerkinElmer
Sucrose	Sigma
TEMED	Biorad
Thenoyltrifluoroacetone	Sigma
Tri-n-octylphosphinoxide	Merck
Triton® X-100	Sigma
Trizma® base	Sigma
Trypan blue stain, 0.4%	Invitrogen
Trypsin, 100x (for cell culture)	Invitrogen
Trypsin (for analysis)	Sigma
Tween 20, 40	Sigma

Kit	Supplier
DLR™ Assay System	Promega
HiSpeed MIDI Kit	Qiagen
pcDNA™3.1 Directional TOPO® Expression Kit	Invitrogen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
ViaLight® Plus Kit	Lonza

Equipment	Supplier
Autoclave	Technomara
CO_2 cell culture incubator	Sanyo
Columbus 12 Channel Head 96 well Strip plate Washer	Tecan
Curix 60 tabletop processor	AGFA
DELFIA® Plate Shaker	PerkinElmer
Eclipse TS100 microscope	Nikon
FPLC	Amersham

ImageScanner	Amersham
Mastercycler Personal	Eppendorf
Mithras LB 940 Luminometer	Berthold Technologies
Neubauer counting chamber	VWR
Orbital Shaker Incubator	TEQ
Rocky Rocking platform	Biometra
Thermomixer 5436	Eppendorf
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell	Biorad
Typhoon 8600 Variable Mode Imager	Amersham
VICTOR3™ Multilabel Counter	PerkinElmer

Consumable	**Supplier**
96-well flat bottom cell culture plate, white	Nunc
96-well flat bottom microtiter plate, clear	Nunc
Blotting paper	Amersham
Cell culture dishes (75 cm2 flasks, 6/12/24-well plates)	Becton Dickinson
Cellulose nitrate filter, 8 µm	Sartorius
Filter, 0.2 µm	Schleicher & Schuell
miniPERM cell culture module	Greiner Bio-One
NHS-activated Sepharose 4 Fast Flow resin	Amersham
Nitrocellulose membrane	GE Healthcare
Petri dish	Roth
Roti-Spin MINI 3 columns	Roth
Self-adhesive film	Nunc
Superdex 75 column	Amersham

Software	**Supplier**
GraphPad Prism 5.01	GraphPad Software
FASTA online sequence comparison service	University of Virginia
MagicScan32 V4.5	Umax
MicroWin 2000	Berthold Technologies
MS Office	Microsoft

Multicalc program	PerkinElmer
PeptideCutter	Swiss Institute of Bioinformatics
RasMol Version 2.4.7.2	RasWin Molecular Graphics
Redasoft Visual Cloning 2000 v.1.0	Redasoft

IV. Primers 5′ → 3′, ordered from Invitrogen (DNA oligos, 25 nmole scale, desalted)

hGH-fwd	CAC CAT GGC TAC AGG CTC CCG
hGH-rev	CTA GAA GCC ACA GCT GCC C
R77C-fwd	CCA ACC TAG AGC TGC TCT GCA TC
R77C-rev	GAT GCA GAG CAG CTC TAG GTT GGA T
D112G-fwd	ACA GCA ACG TCT ATG GCC TCC TA
D112G-rev	TAG GTC CTT TAG GAG GCC ATA GA
C182A-rev	CTA GAA GCC ACA GCT GCC CTC CAC AGA GCG AGC CTG CA
C189A-rev	CTA GAA GCC AGC GCT GCC CTC
Δ188-190-rev	CTA GAA GCC CTC CAC AGA GCG GCA CTG CA
T7 (fwd)	TAA TAC GAC TCA CTA TAG GG
BGH-rev	TAG AAG GCA CAG TCG AGG

V. pcDNA3.1-GH (wt) sequence: recognition sequences for T7 (precedes insert) & BGH-rev (after insert) are in italics and underlined, insert is bolded and the signal sequence is in addition underlined. CACC right before the signal sequence is necessary for directional TOPO cloning.

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCAT
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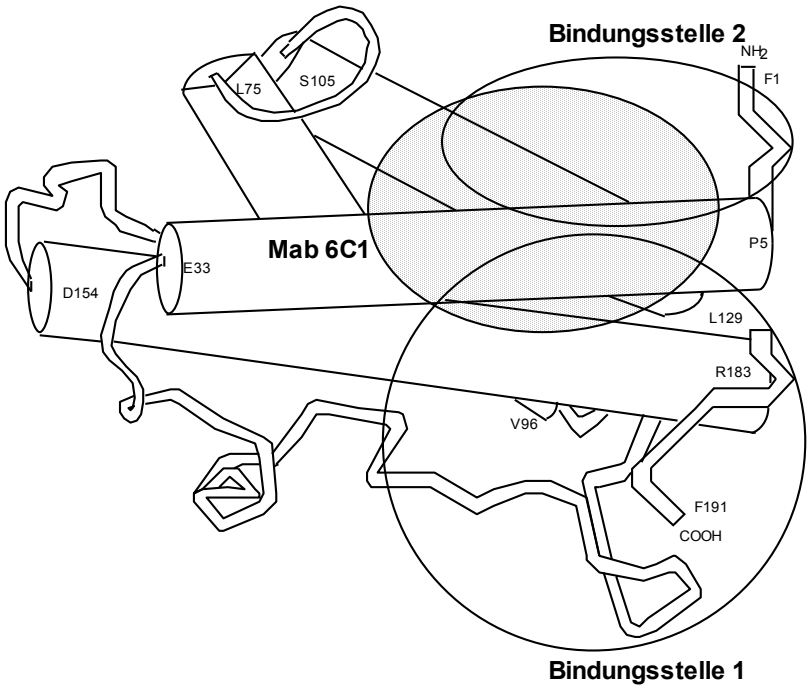
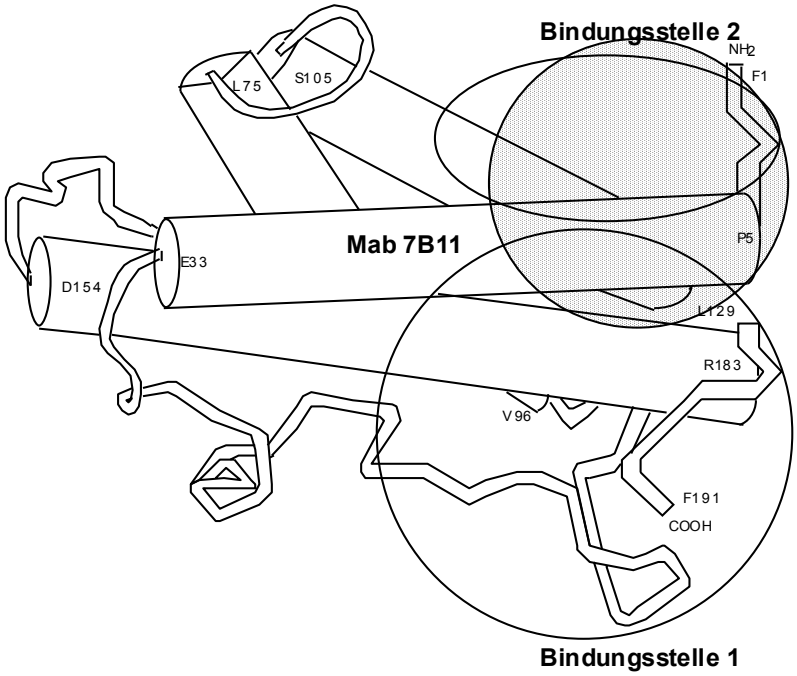
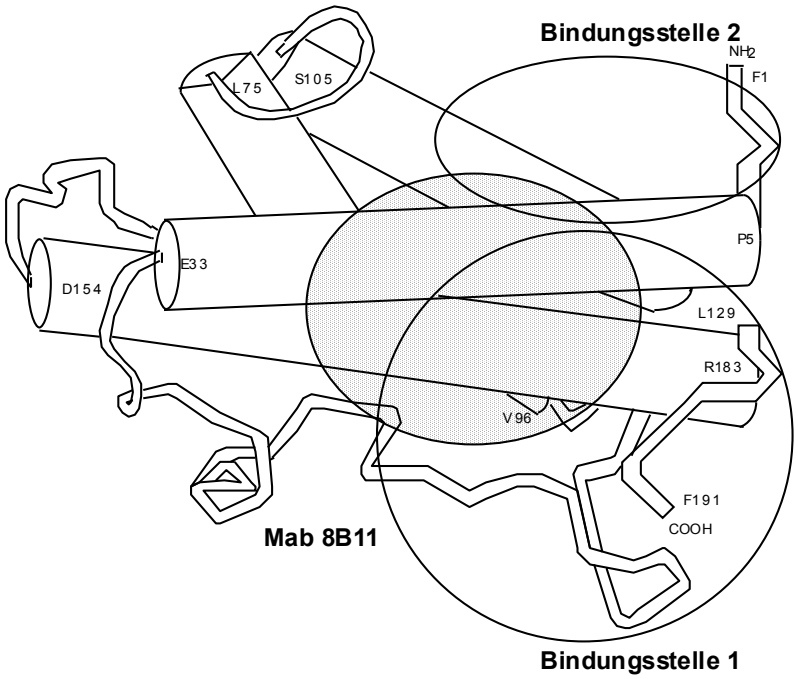
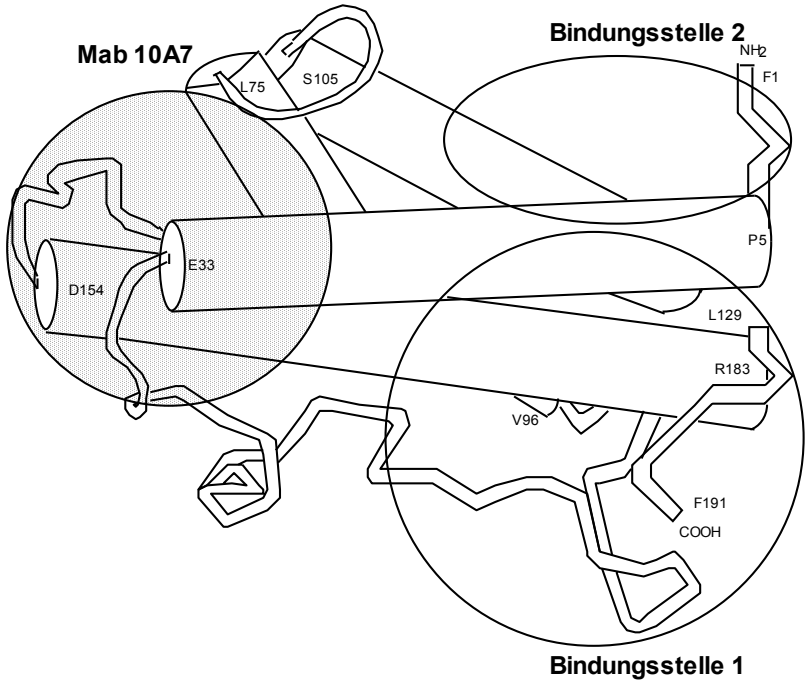
VI. Binding epitopes of in-house mAbs [Wu, 1998]. Bindungstelle is German for binding site.



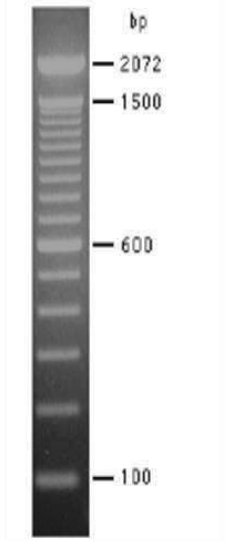



Bindungsstelle 2
NH2
F1
L75
S105
P5
E33
D154
L129
R183
V96
F191
COOH
Mab 8B11
Bindungsstelle 1


Mab 10A7
Bindungsstelle 2
NH2
F1
L75
S105
P5
E33
D154
L129
R183
V96
F191
COOH
Bindungsstelle 1

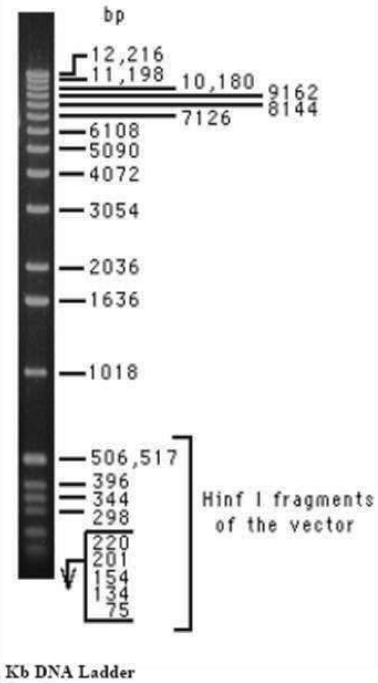
VII. DNA Ladders [Invitrogen]. 0.5 µl Low DNA Mass Ladder contains following amounts of DNA per band: 2000 bp/25 ng, 1200 bp/15 ng, 800 bp/10 ng, 400 bp/5 ng, 200 bp/2.5 ng, 100 bp/1.25 ng.

100 bp ladder



100 bp DNA Ladder
0.5 µg/lane
2% agarose gel stained with ethidium bromide.

1 kb ladder



1 Kb DNA Ladder
0.5 µg/lane
0.9% agarose gel
stained with ethidium bromide

Low DNA mass ladder

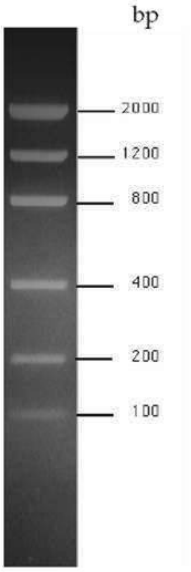


Figure: 2.0% agarose gel loaded with 4 µl of Low DNA Mass Ladder stained with ethidium bromide.

Publications

- ✓ Junnila R, Strasburger CJ, Wu Z (2008): Study of dimer and oligomers of human growth hormone. 10th European Congress of Endocrinology (ECE), Berlin, Germany
- ✓ Junnila R, Strasburger CJ, Wu Z (2008): Study of dimer and oligomers of human growth hormone. Bregenz Summer School on Endocrinology, Bregenz, Austria
- ✓ Junnila R (2008): The structural and functional role of the C-terminal disulfide bridge of human growth hormone. Young Active Research, Annual Meeting, Cologne, Germany (**oral presentation**)
- ✓ Junnila R, Halilovic A, Wobst H, Wricke S, Kaiser S, Strasburger CJ, Wu Z (2008): The structural and functional role of the C-terminal disulfide bridge of human growth hormone. 13th International Congress of Endocrinology (ICE), Rio de Janeiro, Brazil
- ✓ Junnila R, Braig F, Piilonen K, Strasburger CJ, Wu Z (2009): Characterization of growth hormone mutants R77C and D112G found in patients with retarded growth. 11th European Congress of Endocrinology (ECE), Istanbul, Turkey
- ✓ Junnila R, Halilovic A, Wobst H, Kaiser S, Wricke S, Biebermann H, Strasburger CJ, Wu Z (2009): Structural and functional role of the C-terminal disulfide bridge of human growth hormone. Bregenz Summer School on Endocrinology
- ✓ Wu Z, Devany E, Balarini G, Junnila R, Bidlingmaier M, Strasburger CJ (2010): Specific monoclonal antibodies and ultrasensitive immunoassays for 20K and 22K human growth hormone. ***Growth Horm IGF Res*** 20(3):329-44
- ✓ Junnila R, Strasburger CJ, Wu Z (2010): Structural and functional role of the C-terminal disulfide bridge of human growth hormone. The 5th International Congress of the GRS and IGF Society, New York, New York, USA (**oral presentation**)

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel „*In vitro* characterization of human growth hormone mutants“ selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Desweiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsverordnung. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrades.

Berlin den 30. September, 2010

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Riia Junnila