In vitro analysis of hGH secretion in the presence of mutations of amino acids involved in zinc binding

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Abstract

Zinc (Zn$^{2+}$) binding by human GH through amino acid residues His18, His21, and Glu174 has been described as a prerequisite for GH dimerization and storage in secretory granules. Our aim was to investigate in vitro whether disturbed Zn$^{2+}$ binding of mutant GH inhibits wild-type GH (wtGH) secretion and contributes to the pathogenetic mechanisms involved in dominantly transmitted isolated GH deficiency type II. Seven expression vectors harboring mutated human GH cDNAs were constructed in which nucleotide triplets encoding histidine or glutamine at positions 18, 21, and 174 were mutated to triplets encoding alanine: H18A, H21A, G174A, H18A–G174A, H21A–G174A, H18A–H21A, and H18A–H21A–G174A. These vectors were transiently cotransfected with a vector encoding wtGH or were singly transfected into rat pituitary GH 4C1 cells. Plasmids encoding β-galactosidase were cotransfected. After transfection, GH in media and cell extracts was measured using a GH-specific RIA, and results were normalized for transfection efficiency by means of β-galactosidase activity. In comparison with control transfection (wtGH/wtGH set at 100%), GH secretion remained unaffected when coexpressing wtGH and any of the GH mutants in which Zn$^{2+}$ binding was partially or completely prevented. When these mutants were singly expressed, the amount of GH in both media and cell extracts was decreased by about 50% when compared with cells expressing only wtGH. Our in vitro data do not support the hypothesis of disturbed Zn$^{2+}$ binding as a major pathogenetic mechanism in dominantly transmitted GH deficiency.

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Introduction

Zinc (Zn$^{2+}$) plays an important structural role in many proteins including hormones by serving as cross-linking means which bind protein molecules in dimers or big precipitation clusters (Cunningham et al. 1991, Sun et al. 1997). Mutant variants of human insulin which cannot bind Zn$^{2+}$ do not form hexamers and are not properly processed intracellularly, presumably due to the inability of mutant insulin to be concentrated in secretory granules (Carroll et al. 1988, Berg & Shi 1996). Similarly, human prolactin (PRL), which is closely related to human growth hormone (GH), binds Zn$^{2+}$ which is crucial for the intracellular processing and secretion of PRL (Sun et al. 1997, Sankoorikal et al. 2002). Zn$^{2+}$ was shown to be present in human pituitary GH-secretory granules in equimolar amounts with GH, where it forms soluble dimer complexes consisting of two GH molecules and two Zn$^{2+}$ ions (Cunningham et al. 1991). Recombinant human GH is also able to form soluble dimer Zn$^{2+}$ complexes (Yang et al. 2000). Zn$^{2+}$ binding by GH molecules through amino acid residues His18, His21, and Glu174 is a predicted prerequisite for GH dimerization and storage in secretory granules (Cunningham et al. 1991, Yang et al. 2000, Sankoorikal et al. 2002). Thus, it might be hypothesized that loss of affinity of mutant GH to Zn$^{2+}$ could interfere with GH storage and secretion causing disturbed GH secretion as shown for other peptide hormones such as insulin and PRL (Moore & Kelly 1986, Carroll et al. 1988, Berg & Shi 1996, Sun et al. 1997).

Mutations in the intron 3 donor splice site of one GH-1 allele causing skipping of exon 3 are the most frequent causes of the dominantly transmitted isolated GH deficiency (IGHD type II; Cogan et al. 1994, Binder & Ranke 1995). In addition, three missense mutations have been described in IGHD type II patients, which lead to the exchanges of highly conserved amino acids and also suppress wtGH secretion to varying degrees (P89L, V110F, R183H; Duquesnov et al. 1998, Gartner et al. 1998, Binder et al. 2001, Deladoey et al. 2001). These three amino acids involved are located at protruding sites of the tertiary structure of the GH molecule and are possibly engaged in intramolecular interactions of the four α helices or in interactions with other GH molecules (Fig. 1; Uitsch et al. 1994). The cellular mechanisms of the dominant
expression of specific GH-1 mutations in humans, which cause IGHD type II, are still incompletely understood and may be manifold (Binder et al. 1996). Reduction of Zn$^{2+}$ binding and GH dimerization by the mutant GH proteins could be one of these mechanisms, although the amino acids closely involved in Zn$^{2+}$ binding are not directly affected by the mutations found in IGHD type II. Misfolding of mutant GH, however, could result in a molecule which does not exhibit the above amino acids at protruding sites yielding reduced affinity to Zn$^{2+}$.

The aim of this in vitro study using GH4C1 cells was to analyze the importance of intracellular Zn$^{2+}$ binding of GH in view of the exhibition of the dominant negative effect in IGHD type II. The focus was set on regions presumably crucial for aggregation or dimerization, e.g., the histidine and glutamine residues involved in Zn$^{2+}$ binding (His18, His21, Glu174).

### Materials and methods

#### DNA vectors

Point mutations of human GH cDNA were generated using overlap extension PCR technology (Ho et al. 1989). Human wtGH cDNA inserted in a pcDNA3-vector (pwtGH; kind gift from P Dannies, Yale School of Medicine, New Haven, CT, USA) was used as a template. The oligonucleotides used for site-directed mutagenesis are presented in Table 1.

GH-5′–HindIII and GH-3′–XhoI are homologous to the 5′ and 3′ noncoding regions of the GH cDNA including in addition HindIII and XhoI restriction sites respectively.

In a first PCR, amplicons were generated using either GH-5′–HindIII and one of the mutant reverse primers or GH-3′–XhoI primer and one of the mutant forward primers. In a second round of PCR, the primers GH-5′–HindIII and GH-3′–XhoI were used with both amplicons of the first PCR as template. The PCR products were purified using QiAquick Gel Extraction Kit (Qiagen GmbH), cut with restriction enzymes specific for HindIII and XhoI sites, and cloned into the transfection vector pcDNA3.1 (Invitrogen GmbH) opened with HindIII and XhoI. Plasmids were transformed into XL-1 blue supercompetent Escherichia coli cells (Stratagene, Cedar Creek, TX, USA) and screened for efficient cloning by use of restriction enzyme analysis (HindIII and XhoI). Plasmid DNA was isolated from a suitable colony using EndoFree Plasmid Maxi Kit (Qiagen). The presence of the point or deletion mutation and the integrity of the GH cDNA were verified by sequencing (GENterprise GmbH, Mainz, Germany). In addition, we used a previously generated plasmid expressing mutant GH lacking amino acids 32 to 71 which is the mutant GH in most of the cases with IGHD type II (Iliev et al. 2005).

#### Cell culture

GH4C1 cells used for the transfection experiments were purchased from the American Type Culture Collection (ATCC, LGC Promococh, Wesel, Germany). The GH4C1 cell line is a radiation-induced rat pituitary

### Table 1 Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>GH-5′–HindIII</td>
<td>5′-GTT AAG CTT CCT GTG GAC AGC TCA C-3′</td>
<td>5′-AGA CTC GAG TAT TAG GAC AAG GCT GGT-3′</td>
</tr>
<tr>
<td>GH-3′–XhoI</td>
<td>–</td>
<td>5′-AGA CAG GGC GGC GCG GAG-3′</td>
</tr>
<tr>
<td>H18Afor, H18Arev</td>
<td>5′-CGC GCC GGC CTG CTG CAC-3′</td>
<td>5′-CCA GCT GTG CCA GAC GAT G-3′</td>
</tr>
<tr>
<td>H21Afor, H21Arev</td>
<td>5′-CCA GTG TCG TCT GGC ACA GCT G-3′</td>
<td>5′-GGA ATG TGG CGA CTA CCT TGT-3′</td>
</tr>
<tr>
<td>G174Afor, G174Arev</td>
<td>5′-CAA GGT CGC CAC ATT CCT-3′</td>
<td>–</td>
</tr>
</tbody>
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adenoma somatotrope cell line, which produces PRL and rat GH, and proteins are secreted both through the constitutive and the regulated secretory pathways (Scammell et al. 1986). Cells were cultured in DMEM/F-12 (Gibco) supplemented with 15% (v/v) horse serum (Gibco) and incubated at 37 °C and 5% CO2.

**Transient transfection experiments**

The cDNA expression vectors generated, harboring various mutant human GH cDNAs, were in a transient setting, singly transfected or cotransfected with pwtGH and expression vectors for β-galactosidase (pcDNA3.1.V5/His-lacZ, Invitrogen) using the ‘Effectene Transfection Reagent Kit’ (Qiagen). For transfection, 5 × 10^5 cells were seeded in 60 mm poly-D-lysine coated transfection dishes (BD Biosciences, Meylan Cedex, France). Transfection was carried out according to the manufacturer’s instructions 24 h after seeding at ~80% confluency. A total amount of 1 μg DNA was used, the ratio of plasmids encoding wtGH to mutant GH being 1:1 in cotransfection experiments – 0-4 μg pwtGH, 0-4 μg plasmid coding for a mutant GH variant, and 0-2 μg expression vector for β-galactosidase. 48 h after transfection, medium and cells were harvested and cellular proteins were extracted using reporter lysis buffer (400 μl/culture dish) according to the manufacturer’s instructions (Promega).

**GH measurements**

GH values in media and cell lysates were measured using a RIA specific for human GH applying an in-house assay with one polyclonal rabbit anti-hGH antibody against 22 kDa GH as previously described (Hauffa et al. 2004).

**β-Galactosidase activity**

β-Galactosidase activity was determined in an automated luminometer Wallac 1420 Victor^3^ (Wallac Oy, Turku, Finland) using the β-Gal Reporter Gene Assay, chemiluminescent (Roche Diagnostics GmbH).

**Statistical analysis**

Statistical analysis was performed using two-tailed student’s t-test. P<0-05 was considered to indicate significance.

**Results**

The expression vectors that harbored the mutated human GH cDNAs were analyzed by sequencing, which revealed the isolated exchanges of two nucleotide triplets encoding the amino acid histidine at positions 18 and 21, as well as of the triplet encoding the amino acid glutamine at position 174, with a triplet encoding the amino acid alanine. These mutations were predicted to prevent Zn^2+^ binding either partially or completely (Fig. 1).

The mutants were as follows: H18A, H21A, G174A (one Zn^2+^-binding amino acid residue is missing), H18A–G174A, H21A–G174A, H18A–H21A (two Zn^2+^-binding amino acid residues are missing), and H18A–H21A–G174A (all three Zn^2+^-binding amino acid residues are missing). In order to normalize the values obtained by RIA measurements for transfection efficiency, plasmids containing cDNA encoding β-galactosidase were cotransfected and the activity of the gene product was analyzed in cell extracts.

After 48 h of culture, the secreted and stored GH amounts were found to be not different between cells transfected with one of the seven combinations of pwtGH/pmutantGH and cells transfected with pwtGH alone; this was in contrast to the significant reduction of GH observed when the deletion mutant (del32-71) was cotransfected with wtGH (Fig. 2, top). The mean absolute values of RIA-detected GH in media and cell extracts for the pwt/pwt-transfection were 235 and 137 ng/ml respectively while the amount of GH for untransfected cells was around the lower border of the detection limit of our RIA system at 0-23 and 0-51 ng/ml respectively.

However, when these GH mutants were singly expressed, the amount of GH detected both in media and cell extracts decreased by about 50% in comparison with cells expressing only wtGH (P<0.001; Fig. 2, bottom). The GH decrease was found in the presence of all mutants without any significant differences within the group of the different GH mutants.

**Discussion**

The loss of affinity to Zn^2+^ may be one mechanism of the dominant negative effect exerted by some GH mutants, which are monoallelic expressed in dominantly transmitted IGHD type II. Zn^2+^ binding of GH molecules through amino acid residues His18, His21, and Glu174 is regarded as a prerequisite for GH dimerization and subsequently for GH storage in secretory granules (Cunningham et al. 1991, Yang et al. 2000). Here, we examined GH mutants lacking either one, two, or all three of the above crucial amino acids (due to exchange with alanine) in GH4C1 cells, a rat pituitary cell line. When coexpressed with wtGH, the intracellular levels of GH and the amount of GH secreted into the culture medium was not different between cells coexpressing mutant GH and wtGH and

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those expressing only wtGH. Hence, disturbed Zn\(^{2+}\) binding is unlikely to be a relevant mechanism of the dominant negative effect of mutant GH in IGHD type II. However, when mutant GH was singly expressed in GH\(_4\)C\(_1\) cells, we observed a decreased overall production and secretion of GH suggesting a role of Zn\(^{2+}\) binding for GH intracellular trafficking or storage.

In a similar approach, Sun et al. (1997) stably transfected GH\(_4\)C\(_1\) cells with a plasmid encoding mutant human PRL (H27A–PRL) that does not bind Zn\(^{2+}\). They found that the secretion of mutant H27A–PRL was decreased to a highly variable degree, while its intracellular stability was constantly reduced. Concomitantly, rat-GH secretion of these stably transfected GH\(_4\)C\(_1\) cells was reduced. A decrease of H27A–PRL production was also found in transiently transfected GH\(_4\)C\(_1\) cells when transfection efficiency was above 3%.

These observations on human PRL are in agreement with our findings on GH in the same GH\(_4\)C\(_1\) cell line model. In fact, coexpression studies, including both wt PRL and H27A–PRL, were not performed by Sun et al. (1997). Analysis of the aggregation of wt PRL and H27A–PRL in cell-free solutions suggested that PRL, in contrast to GH, does not require a high-affinity Zn\(^{2+}\)-binding site to form aggregates (Sankoorikal et al. 2002). This indicates that, although biologically related, GH and PRL show individual distinct behavior.

The diminished human GH content in cell extracts and medium when GH\(_4\)C\(_1\) cells were singly transfected with one of the different GH mutants cannot be fully explained by decreased immunoreactivity to our polyclonal antibodies of the GH RIA. In a previous study of hGH mutants, we analyzed results of western blot analysis using the polyclonal RIA antibody as well as monoclonal antibodies with RIA measurements and found a good correlation (Iliev et al. 2005). Although these GH mutants were different (i.e., del32-71, C53A, C165A), we suppose that the immunoreactivity measured by the polyclonal RIA truly reflects the total amounts of GH. Therefore, the decrease in GH measured points to a role of Zn\(^{2+}\) binding in GH stability and secretion. This conclusion was also supported by the trend to lower GH levels in the presence of three-point mutations compared with the presence of only one, which may still allow for high-affinity binding to Zn\(^{2+}\). Nevertheless, as coexpression with wtGH did not yield diminished GH levels, there is no indication that loss of high-affinity Zn\(^{2+}\) binding is a major component of the dominant negative effect of mutant GH in IGHD II.

McGuinness et al. (2003) has established the only mouse model for IGHD II where, in addition to the endogenous mouse GH, the human del32-71 GH was expressed. They found severe damage to somatotropic cells of the pituitary gland and panhypopituitarism. Severe cellular damage was not a prominent finding in \textit{in vitro} studies with pituitary cell lines transfected with del32-71 GH (Lee et al. 2000, Iliev et al. 2005). In addition, the panhypopituitarism of the mice was not in line with the main clinical phenotype observed in children with IGHD II, who present with no other hormone deficiency but GH (Binder et al. 2001). However, our recent long-term study on IGHD II indicated that a minority of affected individuals develop additional pituitary hormone deficiencies over time (Mullis et al. 2005). These partly contradictory data suggest limitations of \textit{in vitro} and \textit{in vivo} experiments in the field of the research on IGHD II.

In conclusion, the mutated GH peptides, which poorly bind Zn\(^{2+}\), did not have a major effect on the
secretion of the wtGH isoform; thus, our in vitro data do not support the hypothesis that disturbed Zn\(^{2+}\) binding of mutant GH is involved in the mechanism of dominantly transmitted GHD.

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