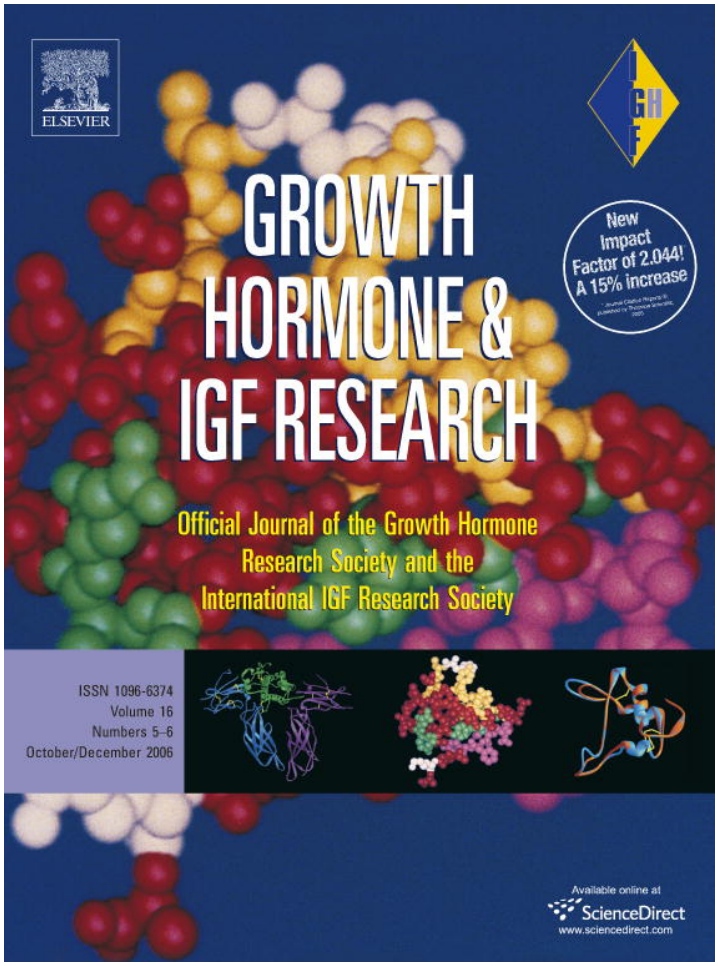

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Large-scale preparation and *in vitro* characterization of biologically active human placental (20 and 22K) and pituitary (20K) growth hormones: Placental growth hormones have no lactogenic activity in humans

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Abstract

Expression plasmids containing DNA sequences optimized for expression in *Escherichia coli* were prepared encoding human pituitary (hGH-N 20K) and placental (hGH-V 20 and 22K) growth hormones. The proteins were expressed in bacteria, refolded and purified to homogeneity by anion-exchange chromatography on Q-Sepharose according to a unique protocol developed for each protein. The yields from 5 l of fermentation culture varied between 400 and 700 mg of electrophoretically pure, over 95% monomeric protein. Circular dichroism (CD) analysis revealed similarity of the purified hGHs' secondary structure to that of the pituitary hGH-N 22K, except for hGH-V 20K, in which the α-helix content was lower. The purified proteins were stable as a 0.1% sterile solution held at pH 10–11 at 4 °C for at least one month. All three purified hGH molecules formed a 1:2 complex with hGH receptor extracellular domain (hGHR-ECD), similar to hGH-N 22K. Binding experiments using hGHR-ECD revealed that the differences between the two 22K variants or between the two 20K variants were not significant, except that hGH-V 20K exhibited slightly lower affinity. Somatogenic activity was tested *in vitro* using FDC-P1 cell lines. Whereas the bioactivity of 22K hGHs and hGH-N 20K in FDC-P1-9D11 cells stably transfected with hGHR was almost equal and two to threefold higher than that of hGH-V 20K, in FDC-P1 3B9 cells stably transfected with rabbit (rb) GHR, the bioactivity of both 20K analogues was significantly (five to ninefold) lower than that of the 22K hormones. The lactogenic activity measured in heterologous assays (Nb2-11C cells and Baf/3 cells stably transfected with the long form of rabbit prolactin receptor) revealed that the activity of hGH-N 20K was close to that of hGH-N 22K in the Baf/3 cells, but 4.5-fold lower in the Nb2 cells. The activity of hGH-V 22K was ninefold less in Nb2 cells and 55-fold less in Baf/3 cells, whereas hGH-V 20K had no lactogenic activity in either bioassay. In contrast, in a homologous lactogenic assay using Baf/3 LP cells stably transfected with hPRLR, the activity of both placental hGHs was nil and the activity of hGH-N 20K was 4.3-fold lower than that of hGH-N 22K. The latter finding raises the question of whether the lack of intrinsic lactogenic activity in the placental hGHs that dominate during pregnancy has any physiological relevance.

Abbreviations: CD, circular dichroism; GH, growth hormone; PRL, prolactin; GHR, growth hormone receptor; ECD, extracellular domain; h, human; o, ovine; r, rat; rb, rabbit.

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1. Introduction

Two genes encode human growth hormone (hGH): hGH-N, which is expressed in the pituitary, and hGH-V, expressed in the placenta. Both hGH-N and hGH-V are 22K, 191-amino acid, single-chain proteins that differ by 13 amino acids. Human GH-V exists as both a glycosylated and a non-glycosylated protein, as it contains an N-linked glycosylation site that is absent in hGH-N. Human GH-V 22K almost completely replaces hGH-N 22K in the maternal circulation by approximately 20 weeks of pregnancy but unlike the latter, it is secreted in a non-pulsatile fashion [1,2]. A cross-sectional study of GH physiology in pregnant women has revealed that in the early stages of pregnancy (up to 15 weeks of amenorrhea), pituitary GH is the main form present in the maternal circulation, displaying a highly pulsatile 24-h serum-concentration profile [1]. From 10 or 20 weeks to term, placental GH gradually replaces pituitary GH, the latter becoming undetectable. Recent studies using transgenic mice engineered to express hGH-V 22K have indicated that it acts like a metabolically active hormone, and its levels in transgenic mice are similar to those found in the third trimester of human pregnancy. Human GH-V 22K interferes with insulin signaling and has been suggested as a likely candidate for the mediation of insulin resistance in pregnancy [3].

Two main approaches to the preparation of recombinant hGH-V 22K have been reported. One, based on expression in *Escherichia coli*, produces hGH-V 22K in its native form, as a soluble protein secreted to periplasm. Expression of hGH-V cDNA in *E. coli* was lower than that of hGH-N cDNA in the same expression system but nevertheless enabled its biochemical and immunochemical characterization [4]. The other method makes use of a eukaryotic system: genes for hGH-N and hGH-V are stably expressed in transfected mouse mammary cells. The biological properties of hGH-N and hGH-V secreted into the medium were examined using rat adipocytes or epididymal fat segments, with methionyl-hGH produced in *E. coli* serving as the reference standard. All three preparations were similar in their ability to bind specifically to intact fat cells and were virtually indistinguishable in their ability to increase glucose oxidation (an insulin-like response), induce refractoriness to insulin-like stimulation, and induce lipolysis in the presence of glucocorticoid [5]. While the somatogenic activity reported in both papers was comparable, lactogenic activity, as determined by rat Nb2 cells possessing prolactin receptor (PRLR), was lower [5]. This finding was confirmed in an additional publication [6]. More recently, it has also been shown that hGH-V produced in eukaryotic cells exhibits signaling pathways similar to hGH-N in IM-9 human lymphocytes and 3T3-F442A preadipocytes, as well as in primary porcine smooth muscle cells. Like hGH-N, signaling by hGH-V could be inhibited by the hGH antagonist, G120K [7].

The gene encoding hGH-N 22K can undergo alternative splicing into a 20K isoform which has 15 deleted amino acids. Human GH-N 20K is produced in the human pituitary [8], and comprises 6–7% of all circulating hGH [9]. Recombinant hGH-N 20K has been prepared as a secreted protein in bacteria [10] and tested in both experimental animals and humans [11]. It has been shown to stimulate linear growth in spontaneous dwarf rats [12] and to induce lipolysis in 3T3L-1 cells expressing hGHR [13]. Taken together, these results and others have demonstrated that recombinant hGH-N 20K has metabolic effects comparable to those of hGH-N 22K in hGH-deficient subjects and that its biological potency is equal to that of hGH-N 22K. Human GH-N 20K could also bind the lactogenic receptors and exhibited lactogenic receptor-mediated activity in Nb2 cells [8]. In adult patients with GH deficiency, hGH-N 20K exhibited metabolic effects comparable to those of hGH-N 22K [14].

In contrast to hGH-N 20K, the expression of hGH-V 20K is still being debated. Using placental cDNA as a template, it has been shown that primary transcripts derived from the hGH-V gene can be alternatively spliced within exon 3 in a manner similar to that observed for the hGH-N gene [15]. The expression of the placental hGH-V 20K transcript seems, however, to vary among different full-term normal placentas and placentas with abnormalities, as the transcript encoding the 20-kDa hGH-V is found in some, but not all placentas [14]. Those authors suggested that this variation in expression might partly explain the previous unsuccessful attempts at detecting this transcript [16–18].

To our knowledge, no recombinant GH-V 20K has ever been prepared. The present work was therefore devoted to its preparation and characterization, as well as to the development of novel protocols aimed at large-scale production of hGH-N 20K and hGH-V 22K, and to a comparative assessment of their bioactivity in homologous and heterologous *in vitro* bioassays. Preliminary results of this work have already been reported [19].

2. Materials and methods

2.1. Materials

Recombinant hGH was a generous gift from Dr. A. Levanon, Biotechnology General Inc. (BTG), Rehovot, Israel, and the extracellular domain of hGH receptor (hGHR-ECD) was purchased from Protein Laboratories Rehovot (Rehovot, Israel). FDC-P1-9D11 and FDC-P1-3B9 cells stably transfected with human or rabbit (rb) GHR, respectively, were obtained from Dr. Mike Waters, and Baf/3 cells stably transfected with the long form of rbPRLR were from Dr. Jean Djiane. An Nb2-11C rat lymphoma cell line was received from Dr. Henri Friesen and Baf/3 LP cells stably transfected with the long form of hPRLR [20] were from Dr. Vincent Goffin. Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA). Highly pure DNA primers were ordered from Sigma Chemical Co. (Rehovot, Israel). RPMI-1640 medium, interleukin-3 (IL3), nalidixic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT) were purchased from Sigma (St. Louis, MO); fetal calf serum (FCS) was from Biolab Co. (Jerusalem, Israel); a Superdex™ 75 HR 10/30 column and Q-Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Antibiotic–antimycotic solution (5×10^4 U/ml penicillin, 50 mg/ml streptomycin, 0.125 mg/ml fungisone), NaCl and Tris base were purchased from Bio-Lab Ltd. (Jerusalem, Israel). Also used were Bacto-tryptone, Bacto-yeast extract, glycerol, EDTA, HCl, Triton X-100, urea (ENCO Diagnostics Ltd., Petah-Tikva, Israel) and NaOH (Frutarom Ltd., Haifa, Israel). Molecular markers for SDS-gel electrophoresis were purchased from BioRad (Hercules, CA). All other materials were of analytical grade.

2.2. Preparation of expression plasmids encoding human pituitary and placental GHs

Synthetic cDNA encoding the sequences of human pituitary 20K GH-N (NCI Gene Database Accession Number NP_072053) and human placental 20K and 22K GH-V (NCI Gene Database Accession Numbers NP_072050 and NP_002050, respectively) was ordered from Entelechon GmbH (Regensburg, Germany). The cDNA was modified to ensure better codon usage and expression in *E. coli* (Table 1). The cDNA in pTOPO was digested with *Nco*I and *Hin*dIII, extracted, and ligated into linearized pMon3401 expression vector. *E. coli* MON105-competent cells were transformed with the new expression plasmid and plated on LB-agar plates containing 75 μg/ml spectinomycin for plasmid selection. Four *E. coli* colonies were isolated and confirmed to contain the respective cDNA, by digestion with *Nco*I/*Hin*dIII restriction enzymes. All of the colonies were positive and one of them was sequenced.

2.3. Expression, refolding and purification of placental hGH-V 20K and 22K

E. coli (strain MON105) cells transformed with the respective plasmids were grown in 2.5-l flasks at 37 °C, (200 rpm) in 500 ml TB medium (1.2% w/v Bacto-tryptone, 2.4% w/v Bacto-yeast extract, 0.4% v/v glycerol in ddH_2O, salts) to an absorbance of 0.9 at 600 nm. Freshly prepared nalidixic acid (2.5 ml, 10 mg/ml) in 0.1 N NaOH was then added. The cells were grown for an additional 4 h, pelleted for 10 min at 7000*g*, and stored at −20 °C. Frozen precipitates from 5 l fermentation were thawed and resuspended in 800 ml cold 10 mM EDTA, pH 8.0, 10 mM Tris–HCl, pH 8.0, in the presence of 0.5 mg/ml lysozyme. Following 30 min incubation on a stirrer at 4 °C, the cells were sonicated for 5 min at the 50%-cycle program and pelleted for 20 min at 10,000*g* (4 °C). The pellet was then suspended in cold ultra-pure water, sonicated and centrifuged as already described. The procedure was repeated twice. The pellet was then suspended in 1% (w/v) Triton X-100, sonicated and centrifuged. The last five or six washes were carried out with cold ultra-pure water; sonication and centrifugation were applied as described. Finally, the pellet containing either hGH-V 20K or 22K protein was solubilized in a total volume of 400 ml containing 4.5 M urea buffered with 40 mM Tris base. Cysteine was added to a final concentration of 50 mM. The pH was increased to 11.0 with NaOH and the clear solution was gently stirred at 4 °C for 1 h. The solution was then diluted with three volumes (1200 ml) of 0.667 M cold arginine, stirred at 4 °C for 2 h and then dialyzed against 5×10 l of 10 mM Tris–HCl, pH 11.0 for 48 h. The resultant solution was then gently mixed with 50 ml Q-Sepharose fast flow anion-exchange resin (30 ml bead volume) pre-equilibrated with 10 mM Tris–HCl, pH 11.0 at 4 °C. Absorbance at 280 and 260 nm was read until it decreased to low values. The Q-Sepharose suspension containing the absorbed protein was then loaded on a column (2.6-cm diameter). Elution was carried out using increasing NaCl solutions in the same buffer (pH 11.0), and 50-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm and monomer content by gel-filtration chromatography on a Superdex™ 75HR 10/30 column. Fractions containing the monomeric protein were pooled, dialyzed against $NaHCO_3$, adjusted to pH 11 to ensure a 4:1 protein-to-salt ratio, and lyophilized.

Table 1
Synthetic DNAs optimized for expression in *E. coli*, used for the preparation of hGH-N 20K, hGH-V 22K and hGH-V 20K[a]

cDNA encoding hGH-N 20K[b]						
CC**ATG**GCTTT	CCCGACCATC	CCGCTGTCTC	GTCTGTTCGA	CAACGCTATG	CTGCGTGCTC	ATCGTCTGCA
TCAGCTGGCT	TTCGACACCT	ATCAGGAATT	TAACCCGCAG	ACTAGTCTGT	GCTTCTCTGA	ATCTATCCCG
ACCCCGTCTA	ACCGTGAAGA	AACCCAGCAG	AAATCTAACC	TGGAACTGCT	GCGTATCTCT	CTGCTGCTGA
TCCAGTCTTG	GCTGGAACCG	GTACAGTTCC	TGCGTTCTGT	ATTTGCTAAC	TCTCTGGTAT	ATGGTGCTTC
TGACTCTAAC	GTATATGACC	TGCTGAAAGA	CCTGGAAGAA	GGTATCCAGA	CCCTGATGGG	TCGTCTGGAA
GACGGTTCTC	CGCGTACCGG	TCAGATCTTC	AAACAGACCT	ATTCTAAATT	CGACACCAAC	TCTCATAACG
ACGACGCTCT	GCTGAAGAAC	TATGGTCTGC	TGTATTGCTT	CCGTAAAGAC	ATGGACAAAG	TTGAAACCTT
CCTGCGTATC	GTTCAGTGCC	GTTCTGTTGA	AGGTTCTTGC	GGCTTC**TGAT**	**GA**AAGCTT	
cDNA encoding hGH-V 22K[c]						
CC**ATG**GCTTT	CCCGACCATC	CCGCTGTCTC	GTCTGTTCGA	CAACGCTATG	CTGCGTGCTC	GTCGTCTGTA
TCAGCTGGCT	TATGACACCT	ATCAGGAATT	TGAAGAGGCT	TATATCCTGA	AAGAACAGAA	ATATTCTTTC
CTGCAGAACC	CGCAGACCTC	TCTGTGCTTC	TCTGAATCTA	TCCCGACCCC	GTCTAACCGT	GTAAAAACCC
AGCAGAAATC	TAACCTGGAA	CTGCTGCGTA	TCTCTCTGCT	GCTGATCCAG	TCTTGGCTGG	AACCGGTACA
GCTGCTGCGT	TCTGTATTTG	CTAACTCTCT	GGTATATGGT	GCTTCTGACT	CTAACGTATA	TCGTCATCTG
AAAGACCTGG	AAGAAGGTAT	CCAGACCCTG	ATGTGGCGTC	TGGAAGACGG	TTCTCCGCGT	ACCGGTCAGA
TCTTCAACCA	GTCTTATTCT	AAATTCGACA	CCAAATCTCA	TAACGACGAC	GCTCTGCTGA	AGAACTATGG
TCTGCTGTAT	TGCTTCCGTA	AAGACATGGA	CAAAGTTGAA	ACCTTCCTGC	GTATCGTTCA	GTGCCGTTCT
GTTGAAGGTT	CTTGCGGCTT	C**TGATGA**AAG	CTT			
cDNA encoding hGH-V 20K[d]						
CC**ATG**GCTTT	CCCGACCATC	CCGCTGTCTC	GTCTGTTCGA	CAACGCTATG	CTGCGTGCTC	GTCGTCTGTA
TCAGCTGGCT	TATGACACCT	ATCAGGAATT	TAACCCGCAG	ACCTCTCTGT	GCTTCTCTGA	ATCTATCCCG
ACCCCGTCTA	ACCGTGTAAA	AACCCAGCAG	AAATCTAACC	TGGAACTGCT	GCGTATCTCT	CTGCTGCTGA
TCCAGTCTTG	GCTGGAACCG	GTACAGCTGC	TGCGTTCTGT	ATTTGCTAAC	TCTCTGGTAT	ATGGTGCTTC
TGACTCTAAC	GTATATCGTC	ATCTGAAAGA	CCTGGAAGAA	GGTATCCAGA	CCCTGATGTG	GCGTCTGGAA
GACGGTTCTC	CGCGTACCGG	TCAGATCTTC	AACCAGTCTT	ATTCTAAATT	CGACACCAAA	TCTCATAACG
ACGACGCTCT	GCTGAAGAAC	TATGGTCTGC	TGTATTGCTT	CCGTAAAGAC	ATGGACAAAG	TTGAAACCTT
CCTGCGTATC	GTTCAGTGCC	GTTCTGTTGA	AGGTTCTTGC	GGCTTC**TGAT**	**GA**AAGCTT	

[a] Initiation and stop codons are in bold letters.
[b] Optimized sequence according to GenBank deposit (NP072053 GH1 202).
[c] Optimized sequence according to GenBank deposit (NP0002050 GH2 217).
[d] Optimized sequence according to GenBank deposit (NP072050 GH2 202).

2.4. Expression, refolding and purification of pituitary hGH-N 20K

A similar solubilization procedure, except for the addition of cysteine to a final concentration of 0.1 mM, was applied. Two hours after adding arginine, the resultant solution was dialyzed against 5×10 l of 10 mM Tris–HCl, pH 10.0 for 48 h and then loaded on a Q-Sepharose (30-ml bead volume) column, pre-equilibrated with 10 mM Tris–HCl, pH 10.0 at 4 °C. Elution was carried out using step-wise increasing NaCl solutions in the same buffer (pH 10.0), and 50-ml fractions were collected.

2.5. Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli [21] in a 15% polyacrylamide gel under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex™ 75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose-column-eluted fraction using TN buffer (25 mM Tris–HCl, 150 mM NaCl, pH 8, 10 or 11).

2.6. Determination of CD spectra and secondary structure

The CD spectra in the wavelength range of 200–240 nm were measured with a Jasco J-810 spectropolarimeter (Tokyo, Japan) using a 0.020-cm rectangular QS Hellma cuvette with a spectral resolution of 1 nm and signal-to-noise ratio of about 1% at 210–220 nm. Solutions were prepared by dissolving the lyophilized samples in 50 mM phosphate buffer, pH 7.6, or 20 mM carbonate–bicarbonate buffer at pH 10.1, followed by centrifugation. Protein concentrations of 40–80 μM were determined spectrophotometrically at 280 nm using light-scattering correction and the respective extinction coefficient at 1 mg/ml. Secondary structures of proteins were determined by applying the procedure and computer program CONTIN developed by Provencher and Glöckner [22] to calculate α-helices, β-sheets, and β-turns as percentage of amino acids involved in these ordered forms. The constraint that the sum of all elements of the secondary structure in a protein must equal unity produced a remainder, which may be interpreted as the content of random coil [23]. In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 21 proteins was employed [24].

2.7. Various binding assays

Determination of hGH:hGHR-ECD complex stoichiometry by gel-filtration chromatography and competitive-binding assays using radioactive ovine (o) GH-N 22K as a ligand were carried out according to procedures described previously [25], except that the addition of mAb F296 was omitted. Iodination of oGH-N 22K was as described previously [26].

2.8. Biological activity in vitro in the FDC-P1-9D11, FDC-P1-3B9, Nb2-11C and Baf/3 bioassays

FDC-P1-9D11 and FDC-P1-3B9 cells were grown as suspension cultures in 75 cm^2 tissue-culture flasks (Nunc, Kamstrup, Denmark). For maximal growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% (v/v) FCS supplemented with antibiotic antimycotic solution and 100 ng/ml recombinant hGH was added to each flask to promote growth. The cells were incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Before seeding, the cells were washed three times with phosphate buffered saline (PBS) and centrifuged at 1000*g*, for 4 min. The cells were then resuspended in RPMI-1640 medium containing 5% (v/v) horse serum. The experiment was performed using 96-well plates (Nunc) in which 0.1-ml suspensions containing 1.5×10^4 cells/well were seeded.

The Nb2 cell line was grown as a suspension culture in 75 cm^2 tissue-culture flasks. For maximal growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% FCS supplemented with antibiotic antimycotic solution. The cells were incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Stationary cultures were obtained by transferring the Nb2 cells into lactogen-free medium in which FCS was replaced by 5% horse serum. The experiment was performed in 96-well plates in which 2.5×10^4 cells/well were seeded.

Baf/3 cells stably transfected with rbPRLR and Baf/3 LP cells stably transfected with hPRLR were grown and treated similarly to FDC-P1 cells except that hGH was replaced by oPRL (100 ng/ml) or hPRL (10 ng/ml), respectively. Prior to the experiment, the cells were washed three times with RPMI-1640 medium containing 5% horse serum and finally suspended in the same medium at 2.5×10^5 cells/ml and seeded in the 96-well plate as already described.

In all assays, increasing concentrations of hGH variants were added and hGH-dependent cell proliferation was determined by MTT assay 48 h after hormone addition. The growth curves were drawn using the Prizm (4.0) nonlinear regression sigmoidal dose-response curve [27] and the EC_{50} values were calculated.

3. Results

3.1. Purification and physico-chemical characterization of hGH-V (20 and 22K) and hGH-N (20K)

All three hGHs were purified by anion-exchange chromatography (see Methods). Fractions containing monomers that were eluted with 100 mM NaCl (hGH-N 20K and hGH-V 20K) or with 200 mM NaCl (hGH-V 22K) were pooled, dialyzed against $NaHCO_3$ adjusted to either pH 11 (hGH-V 20 and 22K) or pH 10 (hGH-N 20K) to ensure a 4:1 protein-to-salt ratio, and lyophilized. The yields varied from 400 to 700 mg from 5 l of bacterial culture. The purity and homogeneity of the purified mutants were documented by SDS-PAGE under reducing conditions. Only one band of ~20 kDa for hGH-N 20K and for hGH-V 20K, and of ~22 kDa for hGH-N 22K were yielded under both reducing and non-reducing conditions (Fig. 1). As expected, in the absence of reducing agent, the mobility of all four proteins was slightly higher, indicating a globular structure. However, all four bands appeared close to or slightly below the 20-kDa protein marker despite the predicted 20- or 22-kDa molecular mass. Since bands of relatively decreased molecular mass were also observed in oGH, human leptin and other proteins (not shown). It was found that this discrepancy origins from the slower mobility of the colored marker. Gel filtration at pH 10 or 11 under native conditions yielded main monomeric peak consisting of at least 95% monomer and corresponding to a molecular mass of ~20 or 22 kDa (Fig. 3, second column from the left). The secondary structures of the three purified proteins, as well as hGH-N 22K were calculated from the CD spectra performed at pH 10.1 and pH 7.6 (for hGH-N 20K and hGH-N 22K), and are shown in Table 2 and Fig. 2. A high content of α-helix (56–58%) for hGH-N 20K, and hGH-V 22K, 5–7% β-sheets and 15% of β-turns and 20–24% of the remainder were similar to hGH-N 22K, indicating proper refolding. No differences between CD spectra of hGH-N 22K, hGH-N 20K at pH 7.6 and pH 10.1 and that of hGH-V 22K at pH 10.1 were observed. In contrast, the α-helix content of hGH-V 20K was lower. Specific extinction coefficients at 280 nm for a 0.1% solution, assuming an extra Ala at the N-terminus, were calculated according to Pace et al. [28], yielding the following values: 1.14, 1.18 and 0.73 for hGH-V 20K, 22K and hGH-N 20K, respectively. The corresponding value used for hGH-N 22K was 0.70. The stability of both hGH-Vs and hGH-N 20K in solution was tested at 4 °C at pHs 11 and 10, respectively. All three proteins, as well as hGH-N 22K, could be stored as sterile 0.1% solutions for 30 days at 4 °C without undergoing any changes in their monomeric content and retaining their activity in the FDC-P1-3B9 bioassay. Both hGH-V 22K and hGH-N

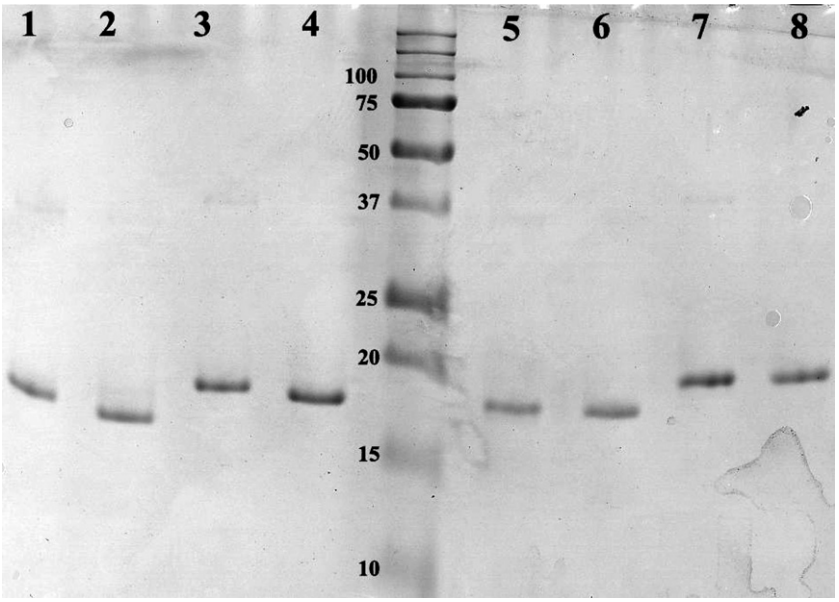

Fig. 1. *SDS-PAGE (15%) of hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K.* Lanes 1 and 5 – hGH-V 20K, 2 and 6 – hGH-N 20K, 3 and 7 – hGH-V 22K, 4 and 8 – hGH-N 22K, middle lane – pre-stained molecular-mass markers (in kDa). Lanes 1–4 are with and lanes 5–8 without β-mercaptoethanol. Aliquots of 1 μg of each protein were applied per lane.

Table 2
Secondary structure of hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K at pH 10.1 or pH 7.6

Secondary structure[a] (%)	hGH-N 22K	hGH-N 20K	hGH-V 22K	hGH-V 20K
α-Helix	58 ± 0.4 (58 ± 0.3)	56 ± 0.4 (51 ± 0.4)	56 ± 0.3	41 ± 0.4
β-Strands	7 ± 0.8 (7 ± 0.4)	6 ± 0.8 (8 ± 0.6)	5 ± 0.7	10 ± 0.7
β-Turns	15 ± 0.5 (15 ± 0.5)	15 ± 0.4 (16 ± 0.3)	15 ± 0.4	17 ± 0.3
Remainder	20 ± 0.9 (20 ± 0.9)	23 ± 0.9 (24 ± 0.7)	24 ± 0.7	32 ± 0.7

[a] Results are given as means ± SD. Errors arose from uncertainty in the fitting of the experimental CD spectrum with the set of standard protein CD spectra in the CONTIN program. Errors in both the CD measurements and the protein concentration determination were not included. The values in parentheses for hGH-N 22K and 20K result from parallel experiments performed at pH 7.6. Both hGH-Vs were poorly soluble at this pH.

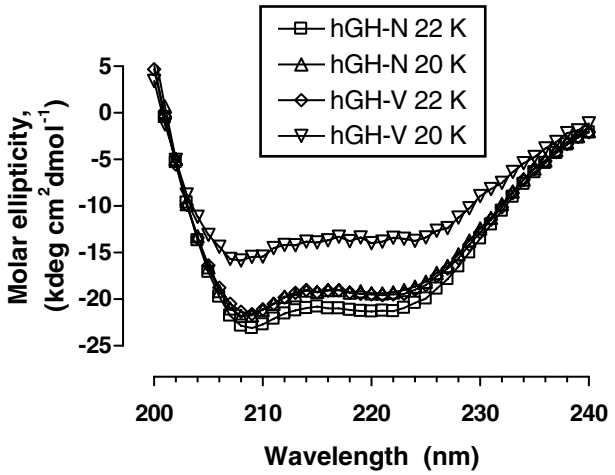

Fig. 2. *CD spectra of purified recombinant hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K.* The proteins were dissolved in 100 mM $NaHCO_3/Na_2CO_3$ buffer pH 10.1. For further details, see text.

20K, as well as hGH-N 22K, but not hGH-V 20K were also stable under those conditions after 6 months. The solubility of hGH-V 22K, and in particular of hGH-V 20K, was highly dependent on the pH and concentration. At pH 11 at 1 mg/ml, both were fully soluble, but their solubility decreased gradually as the pH was lowered to 7.0, depending inversely on concentration.

3.2. Detection of hGH-hGHR-ECD complexes by gel filtration

To characterize the binding stoichiometry between the various hGHs and hGHR-ECD, the respective ligands and hGHR-ECD were mixed in 1:1 or 1:2 molar ratios and separated by gel filtration using an analytical Superdex 75 column to determine the molecular mass of the binding complex under non-denaturing conditions. The experiments were performed using a constant 5 μM of the respective ligand and 10 or 5 μM hGHR-ECD. All four hGHs formed a 1:2 molar ratio complex with hGHR-ECD (Fig. 3). This stoichiometry was evidenced by the appearance of a single main peak for the complex with shorter retention time (11.1–11.3 min), as compared to the higher retention times of hGHR-ECD (13.6–14.1 min) or the hGHs of 22K

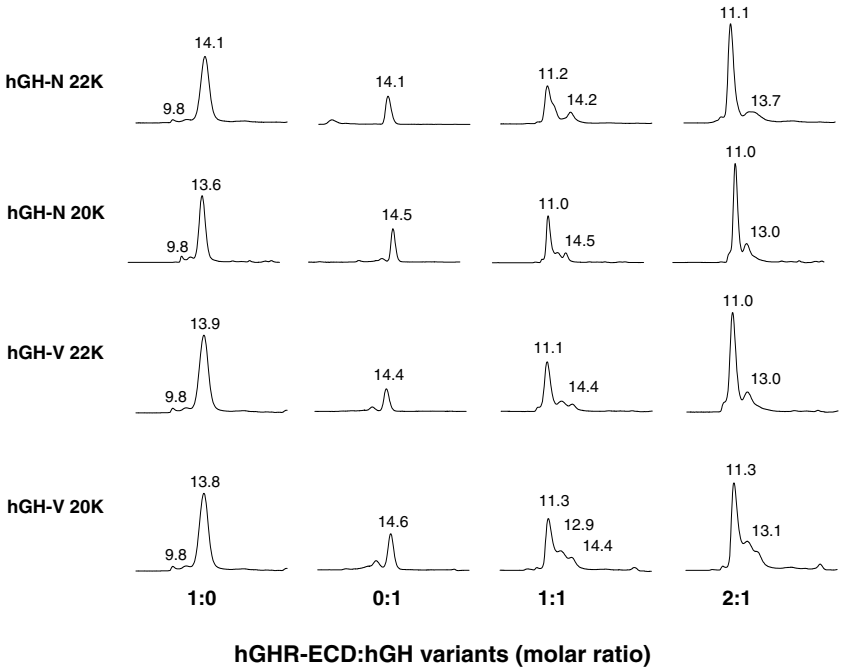

Fig. 3. *Gel-filtration analysis of complexes between hGHR-ECD and hGH-N 22K, hGH-N 20K, hGH-V 22K or hGH-V 20K on a Superdex™ 75 HR 10/30 column*. Complex formation was achieved by 20-min incubation at room temperature in TN buffer using various molar ratios; 200-μl aliquots of the mixture were applied to the column, pre-equilibrated with the same buffer. The numbers above each peak indicate the retention time (RT) in minutes. The final concentrations (5 μM) of the respective hormones and 5 or 10 μM of hGHR-ECD in a complex were used. When the separated proteins were applied the final concentrations of the hGHR-ECD and hGHs were, respectively, 10 and 15 μM. The column was developed at 0.8 ml/min and calibrated with bovine serum albumin (66 kDa, RT = 11.47 min), ovine placental lactogen (23 kDa, RT = 14.2 min) and lysozyme (14 kDa, RT = 21.45 min). The ordinate axis reflects the concentration and the abscissa, the time course. The hGHR-ECD used for the complexes was over 98% monomeric (see left column).

(14.1–14.4 min) or 20K (14.5–14.6 min). The 2:1 peak of the complex appeared also when the components were mixed at a 1:1 molar ratio (retention time 11.07–11.38 min), indicating that the 2:1 complex is a more stable form. A 1:1 complex was almost not visible as a separate peak supporting the conclusion that part of hGH is in 2:1 complex and part of it (characterized by retention time of 14.23–14.57 min) is free.

The calculated molecular mass of the complexes, based on standard markers' peak RTs, were 69–72 kDa, lower than the predicted value of 76–78 kDa for 1:2 complexes of either pituitary or placental hGH 20K or 22K with hGHR-ECD. The differences between the calculated and predicted values are likely related to the differences between the molecular mass and Stockes radius of the complex. Such discrepancies have been reported previously by us [29] and others [30], and are suggested to originate from the more compact structure of the complex.

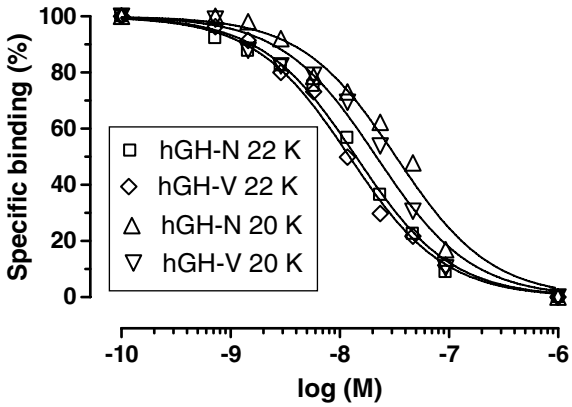

Fig. 4. *Radio-receptor assay using hGHR-ECD.* ^{125}I-human GH (1.5×10^5 cpm/tube) was used as a ligand and hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K as competitors. The results are averages of two or three experiments. The specific binding in two experiments was, respectively, 7.5 and 8.2×10^4 cpm, and was normalized to 100. The non-specific binding was, respectively, 3.6 and 4.0×10^4 cpm. For further details, see text.

3.3. Binding experiments

Iodinated hGH-N 22K served as the ligand in all competitive experiments and the respective non-labeled hGH-N 22K or the other three purified GHs as competitors. Recombinant hGHR-ECD (4 ng/tube) was used as receptor source. The inhibition curves (average of two experiments) are presented in Fig. 4. The IC_{50} values, calculated by Prism 4.0 program [27] (nonlinear regression, best fit for one-site competition), for hGH-N 22K (positive control), and for hGH-V 20K, 22K and

hGH-N 20K were, respectively (in nM, the numbers in parentheses indicate 95% confidence intervals), 14.1 (12.5–16.0), 32.7 (26.0–41.2), 12.3 (10.9–13.9) and 21.7 (17.3–27.2). The differences between the two 22K or between the two 20K variants were not significant. In contrast, the differences between the respective 22K and 20K hGHs showed that the latter exhibit lower affinity.

3.4. Bioassays

Two bioassays measuring the somatogenic activity of the hGH variants using two cell lines stably transfected with human (FDC-P1-9D11) or rabbit (FDC-P1-3B9) GHRs were employed. Results of a typical experiment (out of two to four) are presented in Fig. 5 and Table 3, and show that the activity of both 22K variants was similar and not statistically different. The hGH-N 20K also exhibited similar activity in the 9D11 bioassay but in the 3B9 bioassay, its activity was ~5-fold lower as compared to hGH-N 22K. Similarly the hGH-V 20K, as compared to hGH-N 22K, was ~3-fold lower in the 9D11 bioassay, whereas in the 3B9 bioassay it was ~6.6-fold lower and not significantly different from that of hGH-N 20K.

The lactogenic (PRLR-mediated) activity of hGH-N 20K was similar to that of hGH-N 22K in cells transfec-

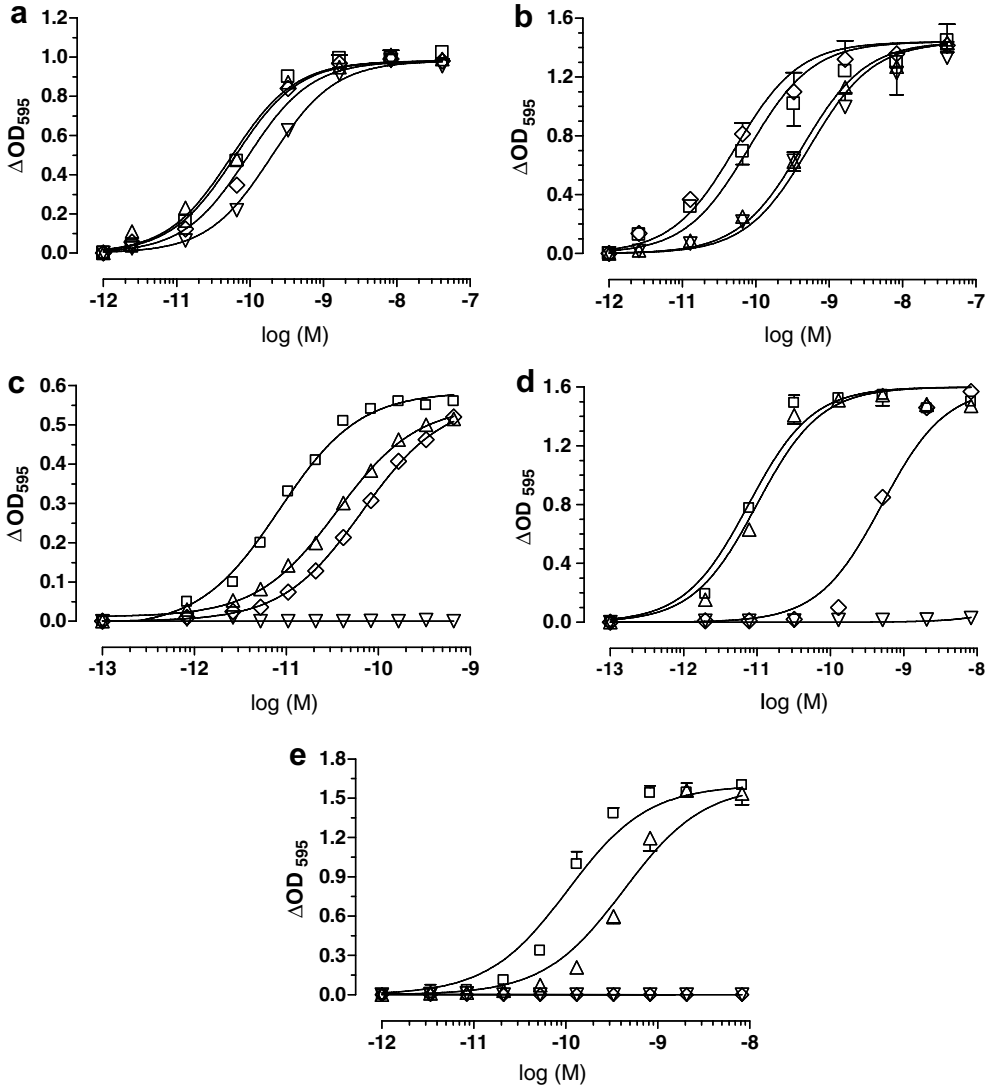

Fig. 5. *Effect of hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K on proliferation of FDC-P1-9D11 (a) and FDC-P1-3B9 (b) cells stably transfected with human or rabbit GHR, respectively, and rat lymphoma Nb2-11C cells (c), Baf13 cells stably transfected with the long form of rbPRLR (d) or Baf13 LP cells stably transfected with the long form of hPRLR (e).* Results of each experiment were normalized to the maximal response and the absorbance in wells not treated with hGH analogues was taken as zero. Representative experiments (out of two to four performed for each analogue) are shown. In all figures, the results are presented as mean ± SEM, but the SEM values were in most cases too small to be visible on the graph. For symbols see Fig. 4 and for other details, see text.

Table 3
The EC_{50} values of hGH-N 22K, hGH-N 20K, hGH-V 22K and hGH-V 20K calculated from bioassays in five different cell lines

Cell line used	Receptor	EC_{50} values (pM) in five different cell lines[a]			
		hGH-N 22K	hGH-N 20K	hGH-V 22K	hGH-V 20K
FDC-P1-9D11	hGHR	62 (45–84)	55 (41–76)	92 (67–126)	194 (164–229)
FDC-P1-3B9	rbGHR	81 (47–139)	427 (335–544)	53 (35–80)	535 (357–801)
Nb_2-11C	rPRLR	8 (6–11)	36 (31–41)	71 (66–78)	None
Baf/3	rbPRLR	9 (5–12)	10 (6–16)	505 (309–830)	None
Baf/3 LP	hPRLR	99 (71–150)	429 (288–637)	None	None

[a] Each value is a mean of two to four experiments; the values in parentheses are 95% confidence intervals of the EC_{50} values.

ted with rbPRLR, but 4.5-fold lower in Nb2-11C cells. The hGH-V 22K analogue, relative to hGH-N 22K, had, respectively, 55- and 9-fold lower activity in both assays, while the hGH-V 20K variant was totally devoid of any lactogenic activity. In contrast, the lactogenic activity mediated through hPRLR (in Baf/3 LP cells) was 4.3-fold lower in hGH-N 20K than in hGH-N 22K, whereas neither placental GH was active at all. Furthermore, to test whether both hGH-V 20K and hGH-V 22K can bind but not activate hPRLR, both proteins were added in 100-fold excess to Baf/3 LP cells stimulated with hGH-N 22K. Neither hGH-V protein exhibited any antagonistic activity, indicating that they do not bind to hPRLRs.

4. Discussion

Recombinant hGH-N 20K, hGH-V 22K and hGH-V 20K were purified to homogeneity, as documented by SDS-PAGE, and contained more than 95% monomeric protein, as evidenced by gel-filtration analyses. The yield was high, 400–700 mg per 5 l of fermentation culture, and the ease of preparation makes it feasible to produce enough material for structural, functional and *in vivo* studies. The secondary structure of hGH-N 20K and hGH-V 22K was similar to that of hGH-N 22K, indicating proper folding, whereas the α-helix content of hGH-V 20K indicated a slightly different secondary structure. The yields reported here seem to be much higher than those reported previously [4–7], and substantially higher than the hGH-V 22K preparation in *Pichia pastoris* reported recently [31].

The binding capacities of the four recombinant hGHs were compared by binding to hGHR-ECD. All four formed 1:2 complexes, confirming the current dogma that the initial step in GH-transduced signal is dimerization of the hGHRs. There were no significant differences in affinity within the two 22K or the two 20K variants, but the latter had approximately twofold higher IC_{50} values in displacing the radioactive ligand.

Since hGH possesses both somatogenic and lactogenic activity, five cell lines were employed for *in vitro* testing. A clear difference between the assays based on FDC-P1 cells stably transfected with rbGHR (3B9) and with hGHR (9D11) was observed. Whereas in the latter assay there were no significant differences between the activities of hGH-N 20K, hGH-N 22K and hGH-V 22K, and the activity of hGH-V 20K was two to threefold lower, in the 3B9 assay, both 22K variants were equally potent, and their activity was markedly higher than those of the two 20K variants, which did not differ between themselves. We feel that this observation is very important because in many reports, hGH activity both *in vitro* and *in vivo* is determined in heterologous rather than homologous bioassays. Furthermore, our results indicate that binding experiments (even those performed with homologous receptor) are not always accurate predictors of biological activity. The assay-dependent outcome was even more pronounced in the three bioassays that measured lactogenic activity. In the most commonly used lactogenic assay, based on rat lymphoma Nb2 cell proliferation, the hGH-N 20K and hGH-V 22K variants were, respectively, 4.5- and 9-fold less potent that hGH-N 22K, as reported by others [31]. In contrast, in Baf/3 cells stably transfected with the long form of rbPRLR, both hGH-Ns were equally potent while the hGH-V 22K was ~55-fold less potent. The hGH-V 20K was devoid of any lactogenic activity in both assays. However, in Baf/3 LP cells stably transfected with hPRLR, hGH-N 20K was ~4-fold less potent than hGH-N 22K, confirming previously reported results [32]. Nevertheless, both placental GHs were totally devoid of any lactogenic activity in the homologous system, and they also lacked any antagonistic properties. As hGH-N 22K is already undetectable by weeks 24–25 of pregnancy [33], our finding raises the question of whether the lack of intrinsic lactogenic activity in the placental hGHs that dominate during pregnancy is of any physiological relevance. It should be noted that human placental lactogen (hPL) shares 85% sequence identity to human growth hormone (hGH) yet hPL binds 2300-fold weaker than hGH to the hGH receptor, while these two hormones have similar affinities for prolactin receptors [34]. Thus it can be speculated that lack of the lactogenic activity in the placental GHs is physiologically compensated by hPL.

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