THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 28, Issue of July 14, pp. 16833–16839, 1995
Printed in U.S.A.

A Growth Hormone Agonist Produced by Targeted Mutagenesis at Binding Site 1

EVIDENCE THAT SITE 1 REGULATES BIOACTIVITY*

(Received for publication, February 16, 1995, and in revised form, May 5, 1995)

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Growth hormone (GH) is believed to signal by dimerizing its receptor through two binding sites on the hormone. Previous attempts to increase the biopotency of GH by increasing its site 1 affinity have been unsuccessful, which has led to a bias toward engineering site 2 interactions in the quest for creation of super agonists. Here we report that increasing site 1 affinity can markedly increase proliferative bioactivity in FDC-P1 cells expressing full-length GHR. In contrast, we find three site 1 mutants with affinities for site one similar to or greater than wild type GH, which have markedly decreased bioactivity. Through crystal structure analysis of the receptor interactive regions of these GH analogues, we are able to suggest why previous mutagenesis on human GH failed to improve biopotency, and thus provide a new avenue for GH and cytokine agonist design.

Growth hormone (GH)[1] has a wide variety of applications both clinically and agriculturally, where it has been found to produce substantial increases in growth rate and protein accretion along with decreases in carcass fat content (1, 2). Improvement in the efficacy of GHs is dependent on an understanding of the molecular basis of GHR (GHR) binding, and the most significant studies delineating this interaction have come from Wells *et al.* (3). In agreement with those studies, the crystal structure of the GH$(GHR)_2$ complex shows the hormone to contain two separate binding sites for the GHR (4). For the propagation of a biological response there is a requirement for sequential binding of the first receptor subunit to site 1 of GH, followed by binding of the second receptor subunit to site 2 to form the GH$(GHR)_2$ complex (5). Because of its role in promoting formation of the biologically effective receptor dimer, the site 2 interaction is thought to be critical in regulation of the biological response (5). Hormone binding site 2 involves residues in the N terminus of GH as well as side chains in helix 3, while site 1 has been proposed to consist of four discontinuous segments, namely the central part of helix 1, residues 38–47 and 54–74, and the C terminus of helix 4 (4, 6). The C terminus of helix 1 has consistently been overlooked as an interactive epitope in mutagenesis studies (7, 8) despite its proximity to receptor 1 in the crystal structure. We have recently shown this region to be an important site 1 binding domain and to be largely responsible for the contrasting Ca^{2+} dependence of binding of non-primate and primate GHs to the rabbit GHR (9).

Despite these advances there have been no reports of GH analogues that show enhanced bioactivity, although a human GH (hGH) that exhibits a 30-fold increased site 1 affinity has been produced (5). The failure of this hGH analogue to show a commensurate increase in efficacy disagrees with conventional pharmacological theory, which predicts that increased receptor occupancy should translate to an increased biological response (10, 11), a concept supported by computer simulations of the biological response to GH binding (12). This disagreement has not been resolved, although it was suggested by Fuh *et al.* (5) that further analysis of the on and off rates of hormone binding may elucidate the issue. Because this high affinity analogue did not have improved biopotency, it was proposed that the rate-limiting step in the biological response was site 2 binding and that in order to create analogues with higher efficacy, modification of binding site 2 was required (5).

In addressing the above issues, we have based our studies on the myeloid line used by Fuh *et al.* (5) but rather than their mG-CSF/hGH chimeric receptor, we have stably transfected the cells with a full-length GHR. This alleviates concerns about the ability of the chimeric mG-CSF/hGHR to accurately represent the physiological signaling mechanism.

Using this assay system, we provide support for our previous findings regarding the importance of the C terminus of helix one as a receptor interactive epitope. In addition, we show that mutations in this region and at a site in the center of helix 4 substantially decrease the biopotency even though they do not adversely affect the affinity of binding to site one. From inspection of the crystal structure, we suggest that two regions of the receptor, the hinge region linking domains 1 and 2 and the final β-turn before the membrane, may be important regions that mediate transmission of the biological signal.

We also report that deletion of the 8 C-terminal residues containing the small disulfide loop results in a pGH analogue with increased site 1 affinity and biological potency. This indicates that improvements to site 1 binding can result in substantial gains in bioactivity, in contrast to previously accepted views (5).

* This work was supported by a Pig Research Development Corporation scholarship (to S. W. R.), a National Health and Medical Research Council R. D. Wright Award (to R. B.), and an Australian Research Council grant (to M. J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GH, growth hormone; bGH, bovine growth hormone; C181S del 184–191 pGH, porcine growth hormone analogue with cysteine mutated to serine at position 181 and with residues 184 to 191 deleted; GHR, growth hormone receptor; hGH, human growth hormone; Me^+, monovalent cation; Me^{2+}, divalent cation; pGH, porcine growth hormone.

MATERIALS AND METHODS

Human GH, pGH, and pGH analogues were expressed in *Escherichia coli* and purified as described in Refs. 9 and 13. L127V[2] bovine GH was a gift from Monsanto (Chesterfield Village, MO), while oPRL-16 was from the National Hormone and Pituitary Program (Baltimore, MD). Far-UV CD spectra were performed on all pGHs, and no measurable change in α-helical content was seen between wild type pGH and pGH analogues. CD spectra are not shown but were identical to those previously reported (13). In addition to DNA sequencing, all analogue pGHs underwent mass spectrometry to verify the incorporation of the desired mutational changes. Protein concentration of mutants was obtained by laser densitometric scan of silver-stained gels and by amino acid analysis.

Establishment of Cell Lines Expressing the Rabbit GHR—FDC-P1 cells are an IL-3-dependent murine myeloid cell line (14), and along with IL-3 were a gift from Andrew Hapel (John Curtin School of Medical Research, Australian Capital Territory, Australia). Cells were routinely passaged in 5% CO_2, 95% O_2 at 37 °C, RPMI 1640 medium supplemented with gentamicin at 1 μg/ml, 5% fetal calf serum (FCS), (from Life Technologies, Inc., Glen Waverly, Victoria, Australia) containing IL-3 at 50 units/ml.

FDC-P1 cells were grown to mid-confluence and transfected by a method modified from Ref. 15. In brief, to a 0.4-cm cuvette was added 5 $\times$ 10^6 FDC-P1 cells in 200 μl of growth media and 0, 5, 10, 15, or 20 μg of pCIS2.RGHR1 DNA (16) in 50 μl of PBS. Cells were electroporated at 960 microfarads and 300 V with a Bio-Rad Gene Pulser apparatus connected to a capacitance extender (Bio-Rad Laboratories Pty. Ltd., North Ryde, Australia). After electroporation the cells were placed in a 25-cm^2 flasks containing 10 ml of fresh growth media supplemented with 50 units of IL-3/ml. Cells were left for 36–48 h (to allow GHR expression), after which they were transferred to growth medium devoid of IL-3 but containing 40 ng/ml hGH. GH selection was continued for 2 weeks with medium changes at 48–72-h intervals. Stably transfected lines were then cloned by repeated limiting dilution in the presence of 40 ng/ml hGH.

MTT Assay—This cell proliferation assay was originally developed for the spectrophotometric quantification of cell growth and viability (17) and so provides a rapid and convenient means by which the proliferation of GH-dependent cell lines can be assessed in response to the addition of mutant GHs.

GH-dependent FDC-P1 cells (clone FDC-P1-RGHR3B, stably expressing the rabbit GHR) were grown to mid-confluence in phenol red-free RPMI 1640 containing 5% FCS, 40 ng/ml hGH, and 1 μg/ml gentamicin and washed by pelleting cells at 500 $\times$ g for 5 min, aspirating the medium, and resuspending in the same volume of sterile PBS. This step was repeated twice to ensure the removal of all free hGH. The cells were then resuspended in phenol red-free RPMI 1640, 5% FCS with 1 μg/ml gentamicin, diluted to a final concentration of 8 $\times$ 10^5 cells/ml, and 50 μl of this suspension was dispensed into each well of a 96-well plate. This was followed by 100 μl of appropriately diluted hormone made up in the same medium. One plate was used to assay each mutant, with the plate divided in half to allow a within plate comparison with wild type pGH. Eight GH concentrations were used per assay, allowing each GH concentration to be assessed in sextuplicate. Plates were placed without lids (to maintain uniform gas exchange) in a humidified chamber for 20–24 h at 37 °C in 5% CO_2, after which 50 μl of 4 mg/ml MTT was added and the plates left for another 3–4 h (18). Assays were terminated by lysing the cells in 120 μl of isopropanol with trituration, and plates were stored in the dark at 22 °C for 10 min before reading the absorbance at 595 nm in a microplate reader (model 450, Bio-Rad).

Some of our pGH analogues (K30Q R34E pGH, K30E R34E pGH, and H170D pGH; numbering system used is in accord with Ref. 19) have been shown to be divalent metal ion (Me^{2+})-dependent in binding to the rabbit GHR (9, 20), and since phenol red-free RPMI 1640 medium was calculated to contain 125 mM Me^+ (120 mM Na^+ and 5 mM K^+) and 0.8 mM Me^{2+} (0.4 mM Ca^{2+} and 0.4 mM Mg^{2+}), it was possible that this was insufficient to achieve maximal binding and therefore a maximal mitogenic response. For this reason each of the Me^{2+}-dependent pGH analogues and pGH were assayed in the presence or absence of an additional 2 mM $MgCl_2$.

Curve fitting was performed by linear regression using the DeltaGraph package for Macintosh desktop computers (Delta Point, Inc., Monterey, CA). The ED_{50} was calculated after subtracting the base line from a maximal GH dose, this being 900 ng/ml for pGH assays and 450 ng/ml for bGH, hGH, and C181S del 184–191 pGH assays (Fig. 3).

[^{3}H]Thymidine Incorporation Assays—Cell proliferation assays using [^{3}H]thymidine were set up in an identical fashion to the MTT assays. The cells were incubated for 14–16 h in the presence of hormone prior to pulsing with 0.5 mCi of [^{3}H]thymidine in 50 μl of RPMI 1640, 5% FCS, 1 μg/ml gentamicin for 5–6 h before harvesting on a Titer Tek cell harvester (Flow Laboratories, ICN Biomedicals Pty. Ltd., Seven Hills, New South Wales, Australia). Glass filters were counted on a Packard 1900 CA β spectrometer with quench correction.

Determination of Affinity Constants for GHs and Characterization of Rabbit GHRs Expressed in FDC-P1 Cells—Affinity values for bGH and all pGH analogues were assessed using rabbit liver microsomes as described in Ref. 9. For determination of hGH affinity for receptors expressed in the FDC-P1 cell line, ^{125}I-labeled hGH was displaced by increasing dilutions of unlabeled hGH.

The number of surface-expressed rabbit GHRs in the transfected line was determined by growing 500 ml of confluent FDC-P1-RGHR3B cells in phenol red-free RPMI 1640 supplemented with 5% FCS, 1 μg/ml gentamicin, and either 40 ng/ml hGH or 100 units/ml IL-3. Cells were pelleted and washed in the same manner as described above. The pellet was finally resuspended in 4 ml of isotonic glucose binding buffer with 20 mM $MgCl_2$ (IGBBM) (22). To 12 $\times$ 75-mm glass tubes was then added 200 μl of IGBBM, 100 μl of ^{125}I-hGH (approximately 200,000 cpm), 100 μl of unlabeled hGH at increasing dilutions, and finally 100 μl (2 $\times$ 10^7 and 2 $\times$ 10^6 cells/ml for those grown in GH and IL-3, respectively) of cells. Assays were shaken gently at 12 °C (to prevent receptor internalization; Ref. 20) for 14 h and terminated by adding 2 ml of ice-cold IGBBM to each tube, followed by centrifugation for 25 min at 4 °C at 1600 $\times$ g. Pellets were counted on an LKB 1274 γ spectrometer. Data analysis and curve fitting were performed as described previously (9, 20). The identical assay procedure as described above was used to bind ^{125}I-hGH to FDC-P1-RGHR3B cells prior to cross-linking, except that Tris was replaced by Hepes in the IGBBM assay buffer and 1.5 $\times$ 10^7 cells were incubated with 1 $\times$ 10^6 dpm of ^{125}I-hGH in a final volume of 2 ml. Cross-linking was performed in the manner described by Barnard and Waters (23).

Crystal Structure Analysis—The complete co-ordinates for the hGH-(hGHR extracellular)$_2$ complex were kindly made available to us by A. De Vos and A. Kossiakoff. These were converted to the pGH(pGHR extracellular)$_2$ complex by use of the homology package, and energy minimized using the Discover program (Biosym Technologies, San Diego, CA) as described in Ref. 9.

RESULTS

Affinity of GH Analogues—The relative affinities of hGH and bGH were 2.4- and 3.5-fold greater, respectively, than pGH (Table I). Of the pGH analogues tested, the K30E R34E pGH, E33K pGH, and C181S del 184–191 pGH analogues showed a greater than 2-fold increase in affinity for the rabbit GHR relative to wild type pGH (2.4-, 2.1-, and 4.8-fold, respectively). The increased affinity of the K30E R34E pGH analogue relative to pGH was still evident when assayed under physiological conditions (1.8-fold increase), but was less than the increase in affinity obtained when binding was performed in 20 mM Mg^{2+}. All other pGH analogues exhibited an affinity similar to wild type pGH (Table I).

Establishment of Stably Transfected Cells—Several clones of FDC-P1-RGHR cells were obtained, with each of these being able to specifically bind GH and showing a dose-response curve almost identical to that of selected FDC-P1-RGHR3B line (data not shown). Affinity cross-linking to the FDC-P1-RGHR3B cells with ^{125}I-hGH revealed bands at around 65 and 116 kDa after subtraction of the hormone component (Fig. 1*A*). Scatchard analysis from 3 independent assays on this clone revealed 186 $\pm$ 12 GHRs/cell with an affinity of 7.6 $\pm$ 1.6 $\times$ 10^9 M^{-1} when cells were supported with GH (Fig. 1*B*). When cells were supported with IL-3, the affinity of hGH for the GHR was similar, 9.69 $\pm$ 2.1 $\times$ 10^9 M^{-1}, however, the receptor number was increased to 2128 $\pm$ 230 receptors/cell (n = 3).

Biopotency of GH Analogues by MTT Assay—Application of the MTT assay to the FDC-P1-RGHR3B cell line provided a fast, sensitive, and non-radioactive method for determining the

[2] Mutations are designated by the wild type residue (in single-letter amino acid code) followed by the position and the mutant residue.

TABLE I

Combined affinity (K_a) and biopotency values for hGH, bGH, pGH, and pGH analogues

Scatchard analysis was used to determine the affinity of each GH and was performed as in Ref. 9. The biopotency of the GHs relative to pGH was determined as described in Fig. 1. One plate was used to assay each mutant, with the plate divided in half to allow a within plate comparison with wild type pGH. Eight GH concentrations were used per assay, allowing each GH concentration to be assessed in sextuplicate. Because some of the pGH analogues have been shown to be Ca^{2+}-dependent (9), additional Mg^{2+} was supplemented to the growth medium to ensure a maximal mitogenic response. Each analogue underwent Scatchard and biopotency analysis a minimum of three times; S.E. is indicated. The values in parentheses are the decreases in bioactivity relative to wild type pGH. *, $p < 0.05$; **, $p < 0.01$ indicate significant change relative to pGH.

Growth hormone	Affinity relative to wild type pGH	Bioactivity relative to wild type pGH	Ratio of bioactivity (with 2 mM $MgCl_2$/without 2 mM $MgCl_2$)
pGH	1.0	1.0	1.05 ± 0.08
bGH	3.5 ± 0.2*	3.01 ± 0.37	ND[a]
hGH	2.4 ± 0.2*	3.80 ± 0.23	ND
P6S pGH	0.7 ± 0.1	0.49 ± 0.07 (2.0)	ND
K30Q R34E pGH	0.9 ± 0.2	0.20 ± 0.02 (5.0)**	0.95 ± 0.26
K30E R34E pGH	2.4 ± 0.4*	0.13 ± 0.01 (7.7)**	0.90 ± 0.10
K139E pGH	1.0 ± 0.2	0.95 ± 0.15	ND
H170D pGH	0.8 ± 0.2	0.33 ± 0.07 (3.0)*	1.37 ± 0.24
K180A pGH	1.0 ± 0.1	1.04 ± 0.03	ND
C181S del 184–191 pGH	4.8 ± 0.6**	5.20 ± 0.6	ND

[a] ND, not determined.

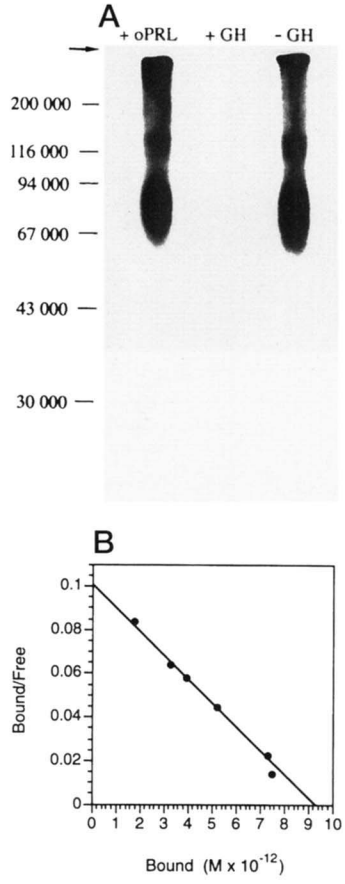

FIG. 1. *A*, autoradiograph of ^{125}I-hGH cross-linked to FDC-P1-RGHR3B cells. ^{125}I-hGH was bound to 1.8×10^8 cells alone or displaced by 2 μg/ml hGH or 1 μg/ml ovine PRL. Cross-linking of GH to the GHR was performed according to "Materials and Methods." Reduced cross-linked cell homogenates were loaded (*arrow* indicates top of resolving gel) and run on a 10% SDS-polyacrylamide gel, with molecular size standards from Bio-Rad or Pharmacia (Amrad Pharmacia, Cannon Hill, Queensland, Australia). The dried gel was exposed to x-ray film for 48 h prior to development of the autoradiograph. *B*, Scatchard analysis of ^{125}I-hGH binding to FDC-P1-RGHR3B cells. Cells were grown to confluence in RPMI 1640 supplemented with 5% FCS and 40 ng/ml hGH. After three washes with PBS to remove hGH, the assay was set up in the manner described under "Materials and Methods," with 1.8×10^7 cells added per assay tube. Each point in the above assay represents the mean of a triplicate determination. The number of receptors per cell was 186 ± 12.

bioactivity of GH analogues. In all cases the base-line (0 ng/ml) and maximal GH response to pGH and the test analogue were virtually identical, enabling an accurate assessment of the ED_{50}. A value of 900 ng/ml was used to obtain the maximal GH response for pGH and helix 1 and 4 mutants, whereas 450 ng/ml was used for bGH, hGH, and the C181S del 184–191 pGH analogue, these values being in the center of the plateau in the bell-shaped curve (Fig. 2). Because each analogue was assayed against wild type pGH in the same plate, interassay variation was not a concern.

The most significant reductions in biopotency of the pGH analogues were seen with K30Q R34E pGH, K30E R34E pGH, H170D pGH, and P6S pGH, which had 5, 7.7-, 3-, and 2-fold reductions respectively in potency compared with wild type pGH. In contrast, bGH, hGH, and C181S del 184–191 pGH showed increased biopotency compared with wild type pGH (Table I). All other pGH analogues had similar biopotency to wild type pGH.

The addition of 2 mM Mg^{2+} to the growth medium elevated nonspecific growth and thus the assay base line at 0 ng/ml; however, the ED_{50} for all of the hormones tested under this condition was not significantly different from that obtained in the control assay performed in the absence of additional Mg^{2+} (Table I).

Cell Proliferation Determined by [^{3}H]Thymidine Incorporation—Over a GH range from 0 to 3×10^5 ng/ml, a bell-shaped dose-response curve was seen with the [^{3}H]thymidine assay (Fig. 2*A*) as reported by Fuh *et al.* (5), similar to that seen with the MTT assay (Fig. 2*B*). This assay also shows the higher potency of hGH compared to pGH, and the fact that inhibition of cell proliferation at the highest hormone concentrations was more pronounced with hGH than with pGH.

DISCUSSION

To facilitate the identification of non-primate GH agonists and antagonists, we have developed a sensitive *in vitro* assay system based on GH-dependent survival of the murine myeloid precursor cell line, FDC-P1. This line normally requires IL-3 for proliferation but has been shown by Fuh *et al.* (5) to convert to GH dependence upon stable transfection with a hybrid receptor consisting of the extracellular domain of the hGHR and the transmembrane and cytosolic domains of the murine G-CSF receptor. Use of this assay system (5) provided support for the proposal that hormone-induced dimerization is required for signal transduction by the GHR, in agreement with the demonstration that GH has two binding sites, each binding a separate GHR molecule (4, 6).

Unfortunately the assay system of Fuh *et al.* (5) did not utilize the full-length GHR, which raises doubts about the validity of derived biopotency data since this construct will not

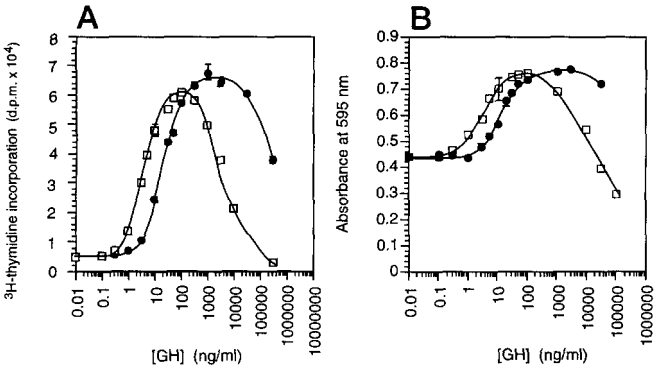

FIG. 2. *A*, bell-shaped dose-response curve for hGH (□) and pGH (●) with FDC-P1-RGHR3B cells using the [^{3}H]thymidine assay. The assay protocol is described under "Materials and Methods." Each point is the mean of a triplicate determination with S.E. indicated. The above curve represents one of three similar assays. *B*, bell-shaped dose-response curve for hGH (□) and pGH (●) with FDC-P1-RGHR3B cells using the MTT assay. The assay was performed as described under "Materials and Methods." Each point in the above curve is the mean of a sextuplicate determination with S.E. indicated. Two additional experiments performed in the same manner gave nearly identical dose-response curves.

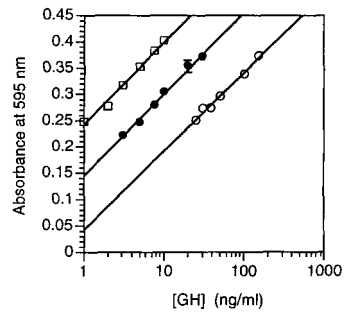

FIG. 3. **Transformed data from MTT assay showing difference in biopotency between pGH (●), C181S del 184–191 pGH (□), and K30E R34E pGH (○) analogues.** A protein concentration correction based on laser densitometry of silver-stained gels and amino acid analysis (9) was applied to these results for calculation of biopotencies. Curve fitting was performed by linear regression using the DeltaGraph package for Macintosh desktop computers (Delta Point, Inc.). The ED_{50} was calculated after subtracting the base line from a maximal GH dose, this being 900 ng/ml for pGH assays and 450 ng/ml for bGH, hGH, and C181S del 184–191 pGH assays. This result was typical of three preparations of the C181S del 184–191 pGH analogue and two preparations of wild type pGH.

express the physiological signaling molecule (*i.e.* does not include the GHR cytoplasmic domain). Moreover, this assay system is restricted to use with primate GHs as a result of the inability of non-primate GHs to bind to primate GHRs. By developing a bioassay system based on the full-length rabbit GHR, we have overcome these limitations. Our stably transfected FDC-P1-RGHR cells have 186 ± 12 receptors/cell when grown in GH and 2128 ± 230 receptors/cell when grown in IL-3. The latter number is approximately 2 times greater than the number of expressed hybrid receptors reported by Fuh *et al.* (5). Affinity cross-linking of the rabbit GHR expressed in this cell line revealed 65- and 116-kDa bands (Fig. 1*A*), a result consistent with the presence of cleaved GH-binding protein and full-length GHR (24). Scatchard analysis (Fig. 1*B*) showed the affinity of hGH for these receptors to be $7.6 \pm 1.6 \times 10^9\ M^{-1}$ and $9.6 \pm 2.1 \times 10^9\ M^{-1}$ when the cells were grown in GH and IL-3, respectively, similar to the values of Gobius *et al.* (22) and Leung *et al.* (16), who transiently expressed the identical rabbit GHR construct in COS-7 cells.

We have used the MTT cell proliferation assay, which allows rapid and convenient spectrophotometric quantification of cell growth and viability (17), to compare the biopotency of our pGH analogues with wild type pGH (Table I). Contrary to the view of Fuh *et al.* (5), a marked gain in biopotency can be achieved through higher affinity site 1 interaction, as evidenced by the C181S del 184–191 pGH analogue. It was observed in the alanine-scanning mutagenesis study of Cunningham and Wells (8) that alanine substitution at Glu-186 and Ser-188 increased affinity, while alanine substitution at Gly-187 decreased it. These results indicate that smaller side chains at this location are more advantageous for receptor binding, and that larger residues cause steric hindrance. Accordingly, Seely *et al.* (25) reported that loss of the disulfide bond between Cys-181 and Cys-189 (C181N C189S pGH and a reduced form of pGH) resulted not only in an analogue that was less likely to aggregate, but one that displayed increased affinity for GHRs. We have made a pGH analogue devoid of the 8 C-terminal residues, and also find increased affinity for binding to rabbit liver GHR (4.8-fold, Table I). However, our C181S del 184–191 pGH analogue shows a 5.2-fold increased bioactivity compared to wild type pGH in the cell proliferation assay (Table I and Fig. 3). On the basis of CD spectra, the loss of the C terminus has not caused significant conformational changes in the GH structure (results not shown), while energy minimization calculations on the mutated analogue reveal little predicted change (less than 1Å) in the overall backbone structure, so no conformational change appears to have been induced at binding site 2. In the crystal structure the C terminus is too distant from site 2 to interact directly, so we conclude that increased site 1 affinity increases biopotency, in accord with the theoretical simulations of Ilondo *et al.* (12).

In an attempt to create further pGH analogues with improved biopotency, we targeted P6 because bGH had a 3-fold higher biopotency compared to pGH and this was one of the few side chains that differed from bGH in the N terminus. This mutation resulted in a 2-fold loss in biopotency without adverse affects on site 1 affinity (Table I), indicating this residue is not responsible for the improved potency of bGH. Additionally, our finding is consistent with the observation of Cunningham and Wells (6) that this side chain is a site 2 determinant.

Two of our analogues are seen to have site 1 affinities and biopotencies similar to pGH. The K139E change is in the floppy loop region between helices 3 and 4, while the K180A mutation is at the end of helix 4. Neither of these side chains are in close contact with either GHR1 or GHR2; therefore, these pGH analogues act as convenient controls.

The greatest decreases in biopotency were seen with the K30Q R34E (5-fold) and K30E R34E pGHs (7.7-fold), even though their affinities for the receptor were not reduced. Similarly, the H170D analogue has wild type affinity but a 3-fold reduction in biopotency. These mutations involve interactions with the GHR at site 1 (9). Since the binding assay measures the affinity of binding site 1 only (6), it follows that the loss in bioactivity seen with these mutants must be a result of unfavorable site 2 interactions, induced either by direct interaction with GHR2 or indirectly through a conformational change in the GH/GHR1 complex that is disadvantageous to receptor 2 binding (*i.e.* to receptor dimerization). We do not favor the former of these proposals because analysis of the crystal structure (4) reveals that the closest contact between side chain head groups in the C terminus of helix 1 of GH and GHR2 is 14.1 Å and 12.2 Å between Lys-30 and Arg-34 of pGH and Gln-166 of GHR2, respectively, while His-170 sits in the middle of the site 1 interface (8). These contacts are too distant to have a significant effect on site 2 binding, and since all side chains are solvent exposed, it is unlikely that mutations at these positions would induce a conformational change in the hor-

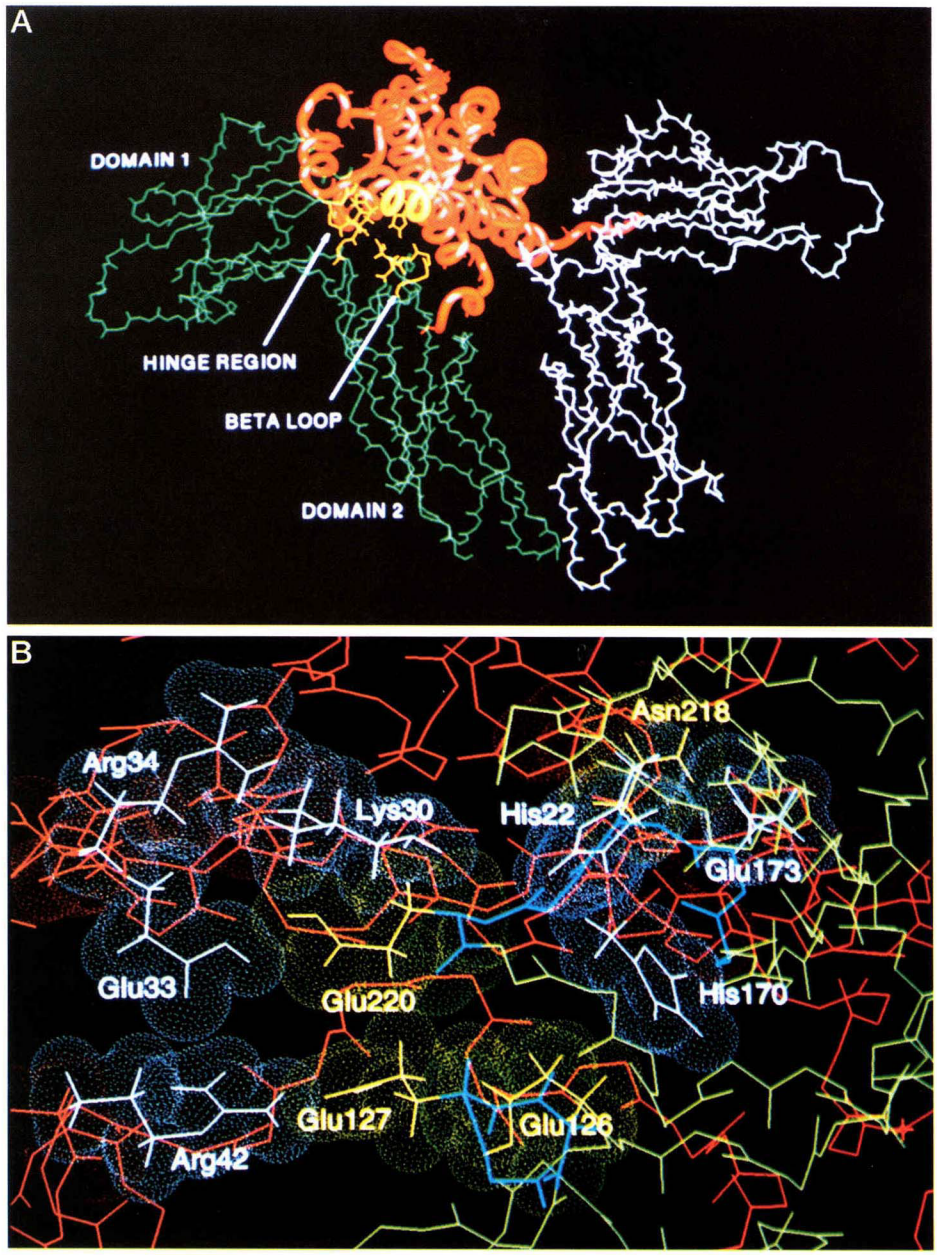

FIG. 4. **Crystal structure (4) of the pGH(pGHR)$_2$ complex detailing.** *A*, pGH (in *orange*) with the C terminus of helix 1 and His-170 highlighted in *yellow*. This region is juxtaposed to the hinge region linking domains 1 and 2 (Glu-126 and Glu-127), and the last β-loop in domain 2 (Glu-220) (all highlighted in *yellow*) of the pGHR1 (in *green*). pGHR2 is in *white*. *B*, examination of the crystal structure of the GH/GHR1 complex reveals two distinct but adjacent clusters binding in the complex, with pGH and pGHR1 side chains having *blue* and *yellow* Van der Waals radii, respectively. The Lys-30-Glu-33-Arg-34-Arg-42/Glu-126-Glu-127-Glu-220 cluster (distances within this cluster are summarized in Ref. 9) is separated from the His-22-His-170-Glu-173/Asn-218 cluster (distances between Asn-218 and His-22 and between Asn-218 and Glu-173 are 4.3 and 3.2 Å, respectively) by a distance of approximately 10 Å. These two clusters are connected through residues Asn-218 and Glu-220, both being found on or near the F/G β-bend in domain 2 of pGHR1. Therefore, there is a strong likelihood that mutations affecting intermolecular interactions in one cluster would have an influence on interactions within the other.

mone. Since energy minimization of these analogues reveals no difference in their overall structures compared to the wild type pGH, direct effects on the site 2 interaction are difficult to envisage, thus unfavorable interactions are most likely to occur through site 1.

We have shown previously that pGH residues Lys-30, Glu-34, and His-170 are in reasonably close contact (between 4 and 7 Å) with Glu-126, Glu-127, and Glu-220 of the GHR (9). There is considerable evidence that these and closely associated side chains may be important mediators of signaling through the GHR. The Glu-126 and Glu-127 side chains are unique in that they are within the 4-residue segment linking domain 1 and 2 of the extracellular portion of the GHR (Fig. 4*A*). This segment was proposed by De Vos *et al.* (4) to be involved in orientating domain 1 and to be the primary reason why domain positioning differs from that seen with the homologous immunoglobulin domains. Indeed, the importance of this hinge region in domain orientation was further highlighted upon the release of the hGH/prolactin receptor crystal structure (26) as superimposing the hGH/hGHR complex onto the hGH/human prolactin receptor complex showed a significant difference in domain orientation of the homologous PRL and GH receptors. A Glu → Tyr change in the hinge region at position 127 was assigned as essentially responsible for the domain shift.

Glu-220 is positioned directly after the β-bend connecting strands F and G in domain 2 (4) (Fig. 4*A*). This β-bend pro-

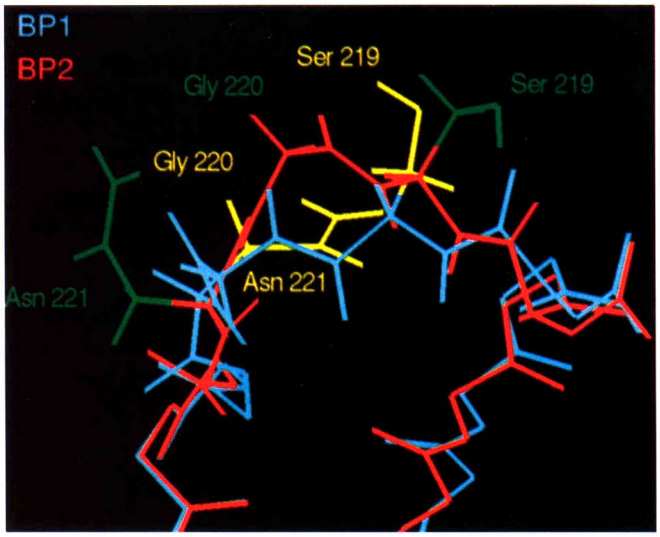

FIG. 5. **Crystal structure of the β-loop linking strands F and G in domain 2 of hGHR 2 (in *red*; side chains in *green*) superimposed onto hGHR 1 (in *light blue*; Ser-219 and Asn-221 side chains in *yellow*).** The distances between α-carbonyl O of residues 217–224 taken from the superimposed hGHR1 and hGHR2 complex are as follows: Arg-217, 0.36 Å; Asn-218, 0.40 Å; Ser-219, 2.35 Å; Gly-220, 3.24 Å; Asn-221, 2.78 Å; Tyr-222, 0.64 Å; Gly-223, 0.39 Å; Glu-224, 0.51 Å. As highlighted previously (4), the loop connecting strands F and G in domain 2 is one of the few regions of the hormone binding interface that is not shared between hGHR 1 and 2. Since this is the case, any difference in the structure within this region is presumably a direct result of GH interaction with GHR1. Thus we predict that the displacement of residues 219–221 and the 180° rotation of Asn-221 is due to contact of hGH side chains His-22 and Glu-173 with Asn-218 of the β-loop.

trudes upward, away from the cell surface and toward the hormone, forming the bottom of a "cupped hands" configuration that holds the GH molecule in the $GH(GHR)_2$ complex. Significantly, there is a difference in β-loop structure that is observed when GHR1 and GHR2 are superimposed (Fig. 5). The β-loop in GHR1 appears to have collapsed as a result of the interaction with GH side chains, principally His-21 and Glu-173, suggesting a hormone-induced conformational change (this is analogous to the conformational change observed in the loop comprising residues 163–168 of the hGHR (4)). Since this β-bend is the last turn before the extracellular domain of the GHR inserts into the plasma membrane, it is plausible that this conformational change is involved in initiation of the biological response.

We suggest that the loss of bioactivity associated with the K30Q R34E, K30E R34E pGH, and H170D pGH analogues is due to unfavorable interactions within either or both of the locations discussed above. Since the GH molecule interacts with GHR1 at the hinge region (Glu-126/Glu-127) and the F/G β-bend (Asn-218 and Glu-220) in the second domain of pGHR1, it seems likely that the measured angle between domains 1 and 2 of GHR1 in the GH/GHR complex is modulated by these interactions (Fig. 4*B*). The angles between the two domains in hGHR1 and hGHR2 differ considerably, and this is a reflection on the different modes of binding of these subunits with the GH molecule. The mutated side chains that interact with the hinge region and β-bend could induce a small movement that manifests a large change in the orientation of receptor domain 2, in much the same way as Tyr-127 is responsible for the difference in domain orientation between the hGH and PRL receptors. A change in the orientation of domain 2 relative to domain 1 in the GHR could affect its ability to dimerize with the identical domain in GHR2. Similarly, an unfavorable displacement of the β-bend could also effect dimerization analogously, or as suggested by crystal structure evidence (Fig. 5), induce an unfavorable conformational change that is transmitted to the submembrane domain, which thereby influences signaling. In support of this proposal, we have recently shown that mutation of Tyr-222 (at the base of the β-bend) to Ala results in complete loss in signal transduction despite only a 5-fold decrease in affinity for hormone (27).

Conformational changes of this type could explain why the high affinity hGH analogue (H21A R64K E174A hGH) of Fuh *et al.* (5) did not have enhanced biopotency, as two of the three mutations (H22A and E173A if using the numbering system of Ref. 28) used to increase site 1 affinity are juxtaposed to the conformation-sensitive region described here. His-22 and Glu-173 are both in close contact with the β-bend in domain 2 of the receptor (Fig. 3*B*). The conversion of these side chains to Ala would decrease the chances of inducing the above mentioned conformational change in the β-bend. Close contact with the β-bend is also an important in the interaction between hGH and the homologous prolactin receptor, as the histidines in helix 1 and Glu-173 in helix 4 of hGH and histidine 218 in the PRL receptor are involved in zinc chelation (26, 29). Accordingly, we propose the mutations used by Fuh *et al.* (5) to make their 30-fold higher affinity hGH analogue were inappropriate for demonstrating the relationship between higher site 1 affinity and biopotency.

In conclusion, our binding and biological activity data show that care must be taken in the design of binding site 1 GH analogues, as it is possible to adversely affect biological activity without loss of site 1 binding affinity. Through analysis of the crystal structure of the $GH(GHR)_2$ complex we suggest that there are two regions on the receptor (the hinge region between domains 1 and 2, and the β-bend) that are conformationally sensitive and important for signal transduction. Confirmation of this hypothesis is required through further mutagenic analysis of the hGH/hGHR interactions or through crystallization of a non-primate GH/GHR complex. Importantly, however, this study shows that it is possible to make analogues of GH that have increased site 1 affinity with a commensurate increase in biopotency. This is consistent with pharmacologic theory and indicates that conventional theory does apply to situations where receptor dimerization is necessary for signal transduction. This realization calls for a re-examination of approaches used in the design of more potent GHs and of members of the structurally homologous cytokine family (28).

Acknowledgments—We thank Ela Knapik for excellent technical assistance in purification of human and porcine GHs and Christine Wells for her tissue culture assistance.

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