In Vitro and in Vivo Studies of Antagonistic Effects of Human Growth Hormone Analogs*

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A bovine growth hormone (bGH) analog, bGH-G119R, has been shown to act as a functional antagonist of GH activity both in vitro and in vivo. In the present study, human (h) GH analogs with alterations in the third α-helix (G120A, G120R) and N terminus (I4A) were generated. These two regions have been reported to form binding site 2 in hGH, which is involved in in vitro dimerization of the GH receptor (GHR). The biological activities of these hGH analogs were tested in vitro by a radioreceptor assay and an assay to test the ability of the molecules to induce tyrosine phosphorylation of a 93-kDa protein in a human lymphocyte line, IM-9. The growth rate of transgenic mice that express different hGH analogs was used as an in vivo test of the activity of the molecules. The results demonstrated that G120R is a potent hGH antagonist both in vitro and in vivo. Transgenic mice expressing G120R demonstrated a growth-suppressed phenotype. However, I4A, which has been demonstrated to be a potent inhibitor of in vitro GHR dimerization, exhibited full growth promoting activity in transgenic mice. Thus, the ability of hGH analogs to induce GHR dimerization in vitro and the ability to promote growth in vivo are not directly correlated.

In the past decade, several achievements have been accomplished in the field of GH research including cloning of the GHR (Leung et al., 1987), determining of the crystal structure of porcine (p) GH and human (h) GH and GH-binding protein (Abdel-Meguid et al., 1987; de Vos et al., 1992), elucidating amino acid residues within GH important for growth promoting activity (Chen et al., 1990, 1991a, 1991b), and defining in vitro interactive domains of GH and GHR (Cunningham et al., 1989; Cunningham and Wells, 1989). Together these results have significantly enriched our knowledge about the structure and function of GH. However, the intracellular biochemical mechanism that is initiated after the binding of GH to its receptor(s) has yet to be defined. Several hypotheses have been proposed for the intracellular mechanism of GH action, which include possible involvement of various kinase systems such as tyrosine kinase (Stred et al., 1990, 1992; Stubbart et al., 1991; Silva et al., 1993), protein kinase C (Tollet et al., 1991; Gorin et al., 1992), and mitogen-activated protein or microtubule-associated protein kinases (Campbell et al., 1992). Also, the findings of nuclear translocation of prolactin (Clevenger et al., 1990) as well as the presence of GH binding protein (GHBP) in the cell nucleus (Lohie et al., 1991) suggests a possible role for intracellular GH in the signaling system.

We have reported previously that the third α-helix of GH is a critical region in the GH molecule which is responsible for its growth promoting activity (Chen et al., 1990, 1991a, 1991b). Mutational analysis of the third α-helix revealed that Gly-119 of bGH is crucial for the activity of this motif (Chen et al., 1991b). Either substitution or deletion mutations of the codon for Gly-119 resulted in a GH analog that acted as a functional GH antagonist both in vitro and in vivo (Chen et al., 1991b, 1991c; Okada et al., 1992). Amino acid sequence comparison demonstrated that Gly-119 of bGH, which corresponds to Gly-120 of hGH, is invariant among GH family members (Table I; Watahiki et al., 1989). Therefore, we postulated that Gly at this position plays a key role in GH growth promoting activity since its minimal side chain size in the middle of the third helix creates a "cleft" which may then interact with a "second target" (Chen et al., 1990, 1991c). We also predicted that substitution of an amino acid with a small side chain such as Ala would have less severe effects on its biological activity as compared with a substitution with an amino acid with a large side chain such as Arg (Chen et al., 1990, 1991a, 1991c).

To test whether our cleft hypothesis proposed for bGH is also true in LGH, we generated hGH analogs with single amino acid substitutions at position 120 (G120A and G120R). We also generated a hGH analog with an amino acid substitution at the N terminus (I4A), since the third α-helix and the N terminus of hGH have been demonstrated to form binding site 2 of hGH (Cunningham et al., 1991; de Vos et al., 1992). The effects of these mutations were examined by in vitro GHR binding assays and an assay to test the ability of these molecules to induce tyrosine phosphorylation of a 93-kDa protein in the human IM-9 lymphocyte line. The growth rate of transgenic mice that express these different hGH analogs was used as an in vivo test of the biological activity of these molecules.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis—All mutated plasmids were derivatives of the parental plasmid, pBG-MT-hGH, which contains the mouse metallothionein I transcriptional regulatory sequence fused to the hGH gene, which contains five exons and four introns. This fusion gene was incorporated into pUC18 at the EcoRI site. An intragenic fragment (from SacI and KpnI) from M13 was introduced into the plasmid, which produces single-stranded DNA for oligonucleotide-directed mutagenesis using standard protocols (Sambrook et al., 1989). All mutations were confirmed by Sanger dideoxy sequence analysis (Sanger et al., 1977).
being harvested for treatment. For in vitro expression, a previously mouse L cell lines that permanently express hGH, G120A, G120R, and described transfection procedure was employed to establish stable gies, Inc.) in a 37 °C, 5% CO₂ incubator. Cells were allowed to reach flasks in RPMI 1640 medium and 10% fetal calf serum (Life Technolo-

Mouse L cells and IM-9 cells were obtained from the ATCC (Rockville, MD). IM-9 cells were grown as suspension cultures in Corning T150 Mouse L cells and IM-9 cells were obtained from the ATCC (Rockville, MD). IM-9 cells were grown as suspension cultures in Corning T150

conditions (Dulbecco's modified Eagle's medium without serum) was collected, and the concentrations were determined by two RIA kits and an insulin-like growth factor I (IGF-I) kit (Nichols Institute Diagnostics) following the manufacturers' recommendations.

Radioimmunassay—RIAs were carried out using two hGH kits (Hybritech and Nichols Institute Diagnostics) and an insulin-like growth factor I (IGF-I) kit (Nichols Institute Diagnostics) following the manufacturers' recommendations.

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Rat, mouse, ovine, bovine, and monkey growth hormone (human placental lactogen) were used. The Nichols kit reacted with three hGH analogs; however, G120R was nondetectable using the Hybritech kit according to the manufacturer's suggestions (Amersham Corp.). Blots were then exposed to x-ray films and developed using standard procedures (Kodak).

Transgenic Mouse Production—The procedure for the production of transgenic mice by direct microinjection of DNA into the male pronucleus of fertilized mouse eggs obtained from B6SJL/J (C57BL/6J x SJL/J) was described previously (Wagner et al., 1981; Chen et al., 1990, 1991a). Transgenic mouse lines were propagated by crossing founder mice with B6SJL/J nontransgenic individuals. DNA extraction from mouse tails and hybridization analyses (slot-blot) were as described (Chen et al., 1990, 1991a). Sera from hybridization-positive mice were tested and quantified for the presence of hGH by RIA.

RESULTS

Expression of hGH and hGH Analogs in Mouse L Cells—Plasmids encoding the mutated and wild type hGH genes (pIG-MT-hGH) were stably introduced into cultured mouse L cells (see “Materials and Methods”). To determine the concentrations of hGH or hGH analogs in conditioned serum-free media, two commercially available RIA hGH kits (Hybritech and Nichols) were used. The Nichols kit reacted with three hGH analogs; however, G120R was nondetectable using the Hybritech kit (Fig. 1A). Western analyses using a polyclonal hGH antibody (BioDesign International) was then used to verify that the same amount of hGH or hGH analogs was used for the RIA analyses (Fig. 1B). These results suggest that the monoclonal antibody used in the Hybritech RIA kit is directed against the third α-helical region of hGH. The results also suggest that substitution of Gly-120 by Ala has significantly less effect on the local conformation of this region as compared with G120R.

### TABLE I

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<thead>
<tr>
<th>Species</th>
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<tr>
<td>Flounder</td>
<td>GH</td>
<td>T-SKLSELKM G LKLIEA</td>
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<tr>
<td>Chicken</td>
<td>GH</td>
<td>VYELKDLEE G IQALMRE</td>
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| Human      | PRL     | TVGQLRLLE G MELVISQ |
| Bovine     | PRL     | TVGQLRLLE G MEMIYGQ |
| Rat        | PRL     | TVGQLRLLE G LKLIEA |
| Mouse      | PRL     | TVGQLRLLE G LKLIEA |
| Salmon     | PRL     | TVGQLRLLE G LKLIEA |

* PRL, placental lactogen.  

** PRL, prolactin.

Cell Culture and in Vitro Expression of hGH and hGH Analogs—Mouse L cells and IM-9 cells were obtained from the ATCC (Rockville, MD). IM-9 cells were grown as suspension cultures in Corning T150 flasks in RPMI 1640 medium and 10% fetal calf serum (Life Technolo-

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Phosphorylation

- Comparison of amino acid sequence alignment among GHs and other members of the gene family

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which is probably because of the size and charge differences of these two amino acids.

**Radioreceptor Binding Assay**—The assay was carried out in a homologous system using $^{125}$I-labeled hGH (DuPont NEN) in the presence of various concentrations of unlabeled hGH or hGH analogs and $1 \times 10^6$ human IM-9 lymphocytes. The results demonstrate that there was no significant difference ($p > 0.05$) in ED$_{50}$ values among hGH (0.22 ± 0.08 nM), G120R (0.32 ± 0.11 nM), I4A (0.39 ± 0.12 nM), and G120A (0.41 ± 0.16 nM; Fig. 2).

**GH Induction of Tyrosine Phosphorylation in IM-9 Cells**—The ability of hGH and the hGH analogs G120R, G120A, and I4A to induce and antagonize a tyrosine phosphorylation event in IM-9 cells was examined. Fig. 3, A and B, shows representative Western blots from three to five separate experiments. Fig. 3A demonstrates that hGH (lanes B–E), I4A (lanes F–I), or G120A (lanes J–M) could significantly induce the tyrosine phosphorylation of a protein with a molecular mass of approximately 93 kDa, whereas G120R was not active in this assay (lanes N–P). The induction of tyrosine phosphorylation is dose-dependent for hGH, G120A, or I4A with the maximum response at the 100 ng/ml range (Fig. 3A, lanes D, H, and L). All three ligands showed self-antagonism at a dose of 1 µg/ml (Fig. 3A, lanes E, I, and M). On the other hand, G120R demonstrated obvious antagonistic effects against hGH at a concentration between 1:1 and 10:1 (Fig. 3B, lane E). The antagonistic effects of G120A or I4A, however, were observed only at 5 µg/ml (Fig. 3B, lanes J and O), which actually reflects the self-antagonistic effects (Fig. 3B, lanes K and P).

**Transgenic Mouse Production**—To test further the in vivo biological activity of these hGH analogs, transgenic mouse lines that express either hGH, G120A, G120R, or I4A were generated. Incorporation of the transgenes into the mouse genome was confirmed by slot-blot analysis (data not shown). Serum hGH or hGH analog levels were determined by RIA. Both DNA- and RIA-positive mice were then monitored for growth. The mean body weights of the transgenic mice (pooled data of founder mice and their F1 and F2 generations) and their nontransgenic littersmates at approximately 2 months of age are shown in Fig. 4A.

Transgenic mice that express G120R exhibited suppressed growth phenotype when it is expressed at relatively high serum levels (Fig. 4A). The degree of growth suppression was directly correlated with the serum levels of G120R (Fig. 5A). These results are similar to those for hGH antagonists expressed in...
transgenic mice (Chen et al., 1990, 1991b, 1991c).

On the other hand, transgenic mice that express I4A and G120A exhibited an enhanced growth phenotype similar to that seen in animals that express wild type hGH (Fig. 4A). The serum levels of I4A or G120A ranged from 50 ng/ml to 5 μg/ml, similar to those found in hGH transgenic mice. There was no correlation between serum hGH, G120A, or I4A levels and the body weight of transgenic mice; i.e. even animals that express low levels of the transgene products (50 ng/ml) resulted in an enhanced growth phenotype. This phenomenon has been reported previously (Palmeter et al., 1983; McGrane et al., 1988; Stewart et al., 1992).

Serum IGF-I Levels in Transgenic Mice—Transgenic mice that express hGH did not show elevated serum IGF-I levels as reported previously (Stewart et al., 1992, Fig. 4B). However, transgenic mice that express G120A or I4A demonstrated a significant elevation of serum IGF-I levels (Fig. 4B). Transgenic mice that express the hGH antagonist G120R showed a significant correlation between levels of G120R and growth phenotype; e.g. the smaller the growth ratio of the mice, the higher the serum G120R levels (Fig. 5A). The IGF-I levels were also negatively correlated to serum G120R levels (Fig. 5B).

DISCUSSION

GH binding to GHR has been reported as a sequential process in which the first binding site of GH interacts with one GHR molecule and then the second binding site interacts with a second GHR, which results in formation of a ternary complex (Cunningham et al., 1991; de Vos et al., 1992; Fuh et al., 1992).
Amino acid residues in the N terminus and within the third α-helix of hGH have been shown to form the second binding site. Specifically, substitution of hGH Ile-4 by Ala (14A) has been shown to decrease the GHR dimer formation by 55-fold and Asp-116 by Ala (D116A) by 6-fold (Cunningham et al., 1991). However, in vitro dimerization activity of G120R was not reported (Cunningham et al., 1991).

A recent report by Silva et al. (1993) demonstrated that hGH induces tyrosine phosphorylation of 93- and 120-kDa proteins in IM-9 cells. They also demonstrated that G120R inhibits this hGH-induced phosphorylation event (Silva et al., 1993). We have recently reported that GH treatment of NIH 3T3-F422A cells resulted in tyrosine-phosphorylated proteins of 95/96 kDa (Wang et al., 1994). It remains to be determined whether the 95/96-kDa proteins in preadipocytes and 93-kDa protein in IM-9 cells are related proteins.

In the present study, hGH analogs with mutations in binding site 2 were generated (G120A, G120R, and 14A). All analogs showed similar affinities to GHR by conventional radioreceptor assay (Fig. 2). However, results of the IM-9 cell tyrosine phosphorylation assay (Fig. 2) were generated (G120A, G120R, and 14A). All analogs behaved differently than G120A or 14A. G120R was inactive in the induction of the 93-kDa protein phosphorylation and exhibited an antagonistic effect (Fig. 3B). However, 14A and G120A exhibited activities similar to wild type hGH (Fig. 3, A and B).

We subsequently generated transgenic mice that expressed these hGH analogs. The results demonstrated that transgenic mice that express G120R exhibited a dose-dependent, growth-suppressed phenotype similar to that seen in hGH-G119R transgenic mice (Chen et al., 1990; Figs. 4A and 5A). On the other hand, transgenic mice expressing 14A or G120A exhibited an enhanced growth phenotype regardless of serum hGH analog levels (Fig. 4A), as one would predict from the results of the tyrosine phosphorylation assay.

There are two discrepancies related to the in vitro dimerization, in vitro tyrosine phosphorylation in IM-9 cells, and in vivo hGH analog results. First, in the tyrosine phosphorylation assay, 14A, G120A, or hGH exhibited self-antagonistic effects at high concentrations (Fig. 3D), similar to what has been reported previously (Silva et al., 1993). In transgenic mice, serum hGH, 14A, or G120A levels reached a relatively high level (<5 µg/ml), which is more than 100-fold excess of endogenous mouse hGH, I4A, or G120A levels reached a relatively high level relative to hGH in induction of the analog showed no significant difference in activity relative to hGH in induction of IM-9 cell protein tyrosine phosphorylation. Thus, the activity of 14A in the in vitro dimerization assay is different from that found in IM-9 cells and in transgenic mice. A possible explanation for the discrepancy may relate to the fact that a bacterial produced, non-glycosylated, non-membrane-bound hGHBP was used in dimerization studies (de Vos et al., 1992) which could behave differently from full-length GHBP found in cultured cells.

Growing evidence suggests that the third helix of hGH might play a critical role in GH signal transduction. For example, a chemically synthesized portion of bGH (amino acid residues 96–133) has been shown to promote several of the biological effects of GH in 3T3-F422A cells while binding to these cells independent of the GH or IGF-I receptors (Sonenberg et al., 1992). Likewise, an 18-mer peptide identical in amino acid sequence to the third α-helix of hGH (110–127) was synthesized and found to stimulate cell division and DNA synthesis in cultured NIH lymphoma cells (Kornberg and Liberti, 1992). Thus, the mechanism by which GH, GH analogs, or GH peptides bind to GHR and stimulate GH-related activities awaits further study.

Finally, IGF-I is generally considered to be a major mediator for animal linear growth. We have demonstrated previously that expression of bGH or bGH analogs in transgenic mice resulted in a growth phenotype that was directly correlated with serum IGF-I levels (Chen et al., 1991c). However, despite their enhanced growth phenotype, hGH transgenic mice do not possess elevated serum IGF-I levels (Fig. 4B). The discrepancy between serum IGF-I levels and an enhanced growth phenotype of hGH transgenic mice has also been observed by others (Stewart et al., 1992). It is interesting to point out that IGF-I levels in both 14A and G120A transgenic mice are elevated (Fig. 4B), which is similar to that seen in bGH transgenic mice. We do not know whether this is a phenomenon related to GHR dimer formation since 14A has been shown to have a 55-fold decreased ability to form GHR dimers (Cunningham et al., 1991). Additional experiments such as quantification of IGF-I binding protein levels or other growth-related parameters in these transgenic mice must be performed to elucidate this observation further.

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REFERENCES


Human Growth Hormone Antagonists


