Functional domains of human growth hormone necessary for the adipogenic activity of hGH/hPL chimeric molecules

Enrique Juárez-Aguilar1, Federico Castro-Muñozledo1,*, Norma E. Guerra-Rodríguez2, Diana Reséndez-Pérez2, Herminia G. Martínez-Rodríguez2, Hugo A. Barrera-Saldaña2 and Walid Kuri-Harcuch1,‡

1Department of Cell Biology, Centro de Investigación y de Estudios Avanzados del I.P.N., Apdo. Postal 14-740 Mexico, D.F. 07000, Mexico
2Department of Biochemistry, Facultad de Medicina de la Universidad Autónoma de Nuevo León, Monterrey, NL, 64460, Mexico
*Author for reprint requests
‡Author for correspondence (e-mail: walid@cell.cinvestav.mx)

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SUMMARY

Genetic analysis through construction of chimeric genes and their transfection in mammalian cells could provide a better understanding of biological functions of native or modified proteins, and would allow the design of new gene constructs encoding peptides that mimic or block ligand interaction with target tissues. To identify the hGH domains responsible for induction of adipose differentiation we constructed hGH/hPL chimeric molecules using homologous DNA mutagenesis, since hGH, but not human placental lactogen (hPL), promotes adipose differentiation in mouse 3T3-F442A cells. We assayed their adipogenic activity in an autocrine/paracrine biological model consisting of transiently transfected 3T3-F442A cells with the chimeric constructs. Plasmid DNAs carrying these constructs were transfected into growing 3T3-F442A cells, and cultures were further maintained for 7 days to differentiate into adipocytes. Secretion of transfected hGH/hPL chimeric proteins into the medium was in the range of 5-25 ng/ml. Adipogenic activity was a property only of those chimeric proteins that contained hGH exon III together with either hGH exon II or hGH IV. Our results also suggest that hGH binding site-2 is composed of two structural subdomains: subsite 2A encoded by exon II of hGH and subsite-2B encoded by exon IV. We also suggest that full adipogenic activity requires the presence of binding site-1 and any of the subsites of binding site-2. This simple autocrine/paracrine biological model of gene transfection allows the analysis of specific biological activity of products encoded by modified genes.

Key words: 3T3-preadipocyte, Adipose-conversion, Adipogenic-activity, Growth hormone, hGH-binding sites, Placental lactogen, Homologous mutagenesis

INTRODUCTION

Human growth hormone (hGH) stimulates tissue growth (Chawla et al., 1983) and promotes differentiation of mesenchymal-derived cell lineages (Ohlsson et al., 1992; Green et al., 1985) including adipocytes (Green et al., 1985; Morikawa et al., 1982). It also modulates metabolism of proteins, carbohydrates and lipids (Chawla et al., 1983).

Enzymatic and chemical cleavage studies showed the existence of molecular domains essential for growth hormone activities (Li and Graf, 1974; Reagan et al., 1975; Yamasaki et al., 1970). Most of the hGH fragments showed decreased or negligible biological activity (Kostyo and Wilhelmi, 1976; Li and Bewley, 1976; Gráf and Li, 1981; Sonenberg et al., 1968; Peña et al., 1980; Aubert et al., 1986; Stevenson et al., 1987). Nevertheless, glucoregulatory effects of peptides such as hGH4-15 (Ng and Harcourt, 1988), hGH6-13 (Ng and Rotstein, 1988), hGH1-43 (Singh et al., 1983) and hGH44-191 (Lewis et al., 1991) suggested that insulin-like activity seems to be associated with the NH2-terminal domain (Stevenson et al., 1987; Salem 1988; Mondon et al., 1988), while diabetogenic effects were associated with the COOH-terminal domain (Lewis et al., 1991). Most evidence on the interaction of hGH with its receptors comes from crystallographic and receptor-binding studies, which do not necessarily reflect the biological activity of hGH domains. The hGH molecule contains two binding sites, 1 and 2, that after interaction with the cell receptors promote their dimerization (Cunningham et al., 1991). We attempted to identify, by genetic analysis, the hGH domains involved in the induction of adipose differentiation.

Adipose differentiation of 3T3-F442A cells depends upon components of the animal serum added to the culture medium (Kuri-Harcuch and Green, 1978). Sera from several species, including the human, contain molecules, amongst them growth hormone, that promote fat cell differentiation; fetal bovine serum is highly adipogenic whereas serum from the domestic cat does not support adipose conversion, although it supports cell proliferation (Kuri-Harcuch and Green, 1978). Therefore,
adipogenic substances can be assayed in 3T3-F442A cells cultured with medium supplemented with serum from domestic adult cat (non-adipogenic medium) (Kuri-Harcuch and Green, 1978). The 3T3-F442A cells have specific receptors to GH and they undergo adipose conversion upon incubation with this hormone (Morikawa et al., 1982, 1984; Nixon and Green, 1983).

When the 3T3-F442A cells undergo adipose conversion, they increase several fold their lipogenic enzyme activities (Kuri-Harcuch and Green, 1977) such as glycerophosphate dehydrogenase (GPD). The expression of transcription factors such as PPARγ and C/EBPα is also induced early during the adipose differentiation, and they regulate the expression of the adipose phenotype (Tontonoz et al., 1994; Clarkson et al., 1995; Hu et al., 1995). GPD is expressed later than PPARγ and C/EBPα, and it is considered to be a primary enzyme in the adipose conversion process; its activity does not depend upon fatty acid synthesis (Kuri-Harcuch et al., 1978) or other changes in the culture medium. The activity of this enzyme increases up to 500-fold during adipose differentiation (Wise and Green, 1979; Kuri-Harcuch et al., 1978), and it is widely used as a sensitive and specific marker to determine the extent of this process (Morikawa et al., 1984; Uchida et al., 1990; Pairault and Green, 1979). Therefore, adipose differentiation of the 3T3-F442A cells is a sensitive and valuable model to study some of the biological effects of GH (Morikawa et al., 1984; Nixon and Green, 1983; Schwartz, 1984; Schwartz and Carter-Su, 1988).

In order to identify the hGH domains involved in induction of adipose differentiation, we took advantage of the homology between hPL and hGH (Bewley et al., 1972; Cooke et al., 1981). Although hPL shares with hGH about 85% identity in amino acid sequence, hPL does not promote tissue growth (Aloj and Edelhoch, 1971) or adipose conversion (Morikawa et al., 1984). We have constructed chimeric molecules between hGH and hPL using homologous DNA mutagenesis (Reséndez-Pérez and Barrera-Saldáña, 1990), and assayed their adipogenic activity in transiently transfected 3T3-F442A cell cultures. We demonstrate that adipogenic activity of the modified gene products depends upon the presence of amino acid sequences encoded by hGH exon III combined with that of either exon II or exon IV of the hGH gene. Our results also suggest that hGH binding site 2 is composed of two structural subdomains: subsite 2A encoded by exon II and subsite 2B encoded by exon IV (see Fig. 5). This type of autocrine/paracrine biological model, depending on cell type, using cells transiently transfected with modified genes is valuable to analyze the biological activity of peptides encoded by such gene constructs.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from Life Technologies (Gaithersburg, MD) and New England Biolabs (Beverly, MA) and used according to the instructions of the manufacturer. DOTAP transfection-reagent was from Boehringer Mannheim GmbH (Mannheim, Germany). Eagle’s medium modified by Dulbecco-Vögt (DMEM) and Opti-MEM™ I were from Life Technologies (Gaithersburg, MD); calf serum was from HyClone Laboratories (Logan, UT). Cat serum was obtained by bleeding adult domestic cats. Recombinant human growth hormone (RhGH) was kindly provided by Genentech Inc. (San Francisco, CA), and human placental lactogen (hPL) was obtained from NIDDK-NIH (University of Maryland School of Medicine). Epidermal growth factor (EGF) was from IMCERA Bioproducts, Inc. (Terre Haute, IN); Insulin, d-biotin, human transferrin, triiodothyronine and bovine serum albumin (BSA) were from Sigma Chemical Co. (St Louis, MO). The Double Antibody Human Growth Hormone and the Coat-A-Count®-HPL radioimmunoassay kits were purchased from Diagnostic Products Corporation (Los Angeles, CA). All other reagents were analytical grade.

Recombinant plasmids

For transfection experiments, we used the pNUT plasmid (a gift from Dr Richard Palmiter, University of Washington at Seattle, WA) (Palmiter et al., 1987), which carries the hGH-N transcriptional unit as the source of hGH. A version of pNUT with its hGH-N gene insert deleted was used as a negative control. The recombinant plasmids were obtained by homologous mutagenesis as described previously (Reséndez-Pérez and Barrera-Saldáña, 1990); we took advantage of the unique homologous restriction sites present in the exon flanking regions of the hGH or hPL genes to replace one or several exons of the hGH gene by the corresponding exon of hPL gene or vice versa (see Fig. 4). Briefly, the recombinant plasmids were constructed by substitution of hGH-N gene sequences with the corresponding homologous regions from either the hPL-2 or the hPL-3 genes, which in spite of minor nucleotide differences code for an identical mature hormone. The fragments of interest were obtained from pNUT, pAVEhGH-N, pAVEhPL-2 and pAVEhPL-3 plasmids (Ramírez-Solís et al., 1990) after digestion with suitable restriction enzymes. Purification of gene fragments, ligations, and bacterial transformations were carried out by standard methods (Sambrook et al., 1989). The hGH/hPL chimeric genes consisted of promoter-less constructs, that replaced the hGH-N gene in the pNUT expression vector previously digested with BamHI and EcoRI; their expression was directed by the metallothionein promoter and an SV40 enhancer. Characteristics and nomenclature used for these genes is summarized in Table 1 and Fig. 4. Recombinant plasmids were first isolated from 3 ml overnight cultures. The identity of the chimeric hGH-hPL chimeric recombinant plasmids was corroborated with different restriction enzymes (Fig. 4) since these constructs were obtained by standard methods of cloning without using PCR to avoid introduction of changes in the DNA sequence (Sambrook et al., 1989). Size of the chimeric hGH/hPL expression products was checked by labeling secreted proteins with 35S)methionine as previously described (Reséndez-Pérez and Barrera-Saldáña, 1990; Reséndez-Pérez et al., 1990). For experiments, plasmids were prepared in large quantities using the Triton X-100 gentle lysis-CsCl gradient procedure (Clewell and Helsinki, 1969).

Cell culture and transfection assay

3T3-F442A cells, kindly provided by Dr H. Green (Harvard Medical School) were plated in Corning tissue culture dishes and maintained as previously described (Salazar-Oblio et al., 1995). For transfection experiments, cells were plated at 700 cells/cm² in DMEM supplemented with 3.5% (v/v) cat serum (non-adipogenic medium) (Kuri-Harcuch and Green, 1978), containing 5 μg/ml insulin and 1.0 μM d-biotin (Kuri-Harcuch et al., 1978) and re-fed every other day. Two-day pre-confluent cultures were changed to serum-free medium (Opti-MEM™ I) plus 55.0 μM 2-mercaptoethanol, containing 1.0 μg/ml plasmid DNA and 3.7 μg/ml DOTAP transfection reagent previously mixed according to the manufacturer’s instructions. After 24 hours incubation, cultures were changed to DMEM containing 1.7%(v/v) cat serum, 0.2%(v/v) calf serum, 5.0 μg/ml insulin, 5.0 μg/ml transferrin, 1 μM d-biotin, 2 mM triiodothyronine, 40 μM 2-mercaptoethanol and 0.01 ng/ml EGF (definitive medium) (Morikawa et al., 1984). Cultures were maintained for 7 days without further
Adipogenic activity of hGH/hPL chimeric proteins

RESULTS

Transient transfection of 3T3-F442A preadipocytes

The 3T3-F442A preadipocytes when incubated with hGH concentrations higher than 0.1 ng/ml undergo adipose conversion (Morikawa et al., 1984) and they increase lipogenic enzyme activities; among them, glycerophosphate dehydrogenase which is a sensitive and specific marker of this differentiation process (Kuri-Harcuch et al., 1978; Wise and Green, 1979; Pairault and Green, 1979). We assayed the adipogenic activity of increasing concentrations of hGH or hPL in 3T3-F442A cells cultured in non-adipogenic medium (Figs 1B and 2). Concentrations of hGH from 0.1 to 100.0 ng/ml promoted adipose conversion with a maximal differentiation obtained at 5.0 ng/ml of hGH (Fig. 2). However, as previously reported, hPL did not promote adipose conversion even at concentrations higher than 100.0 ng/ml of hPL (Fig. 2) (Morikawa et al., 1984). Therefore, we transfected the 3T3-F442A cells with hGH/hPL chimeric genes in an attempt to promote adipose conversion through the action of the secreted chimeric proteins. Cultures of growing 3T3-F442A cells (60% confluent) were transfected with increasing concentrations of GH-N or PL plasmid DNA carrying the genes encoding normal hGH and hPL, respectively. After 24 hours, cells were changed to non-adipogenic (definitive) medium and further maintained for 7 days in culture (Fig. 1A). Transfection of cultured cells with plasmid expressing the hGH gene underwent adipose conversion to levels similar to those found in control cultures maintained with 25 ng/ml of recombinant hGH (Fig. 3; Table 2). In contrast, since hPL is not an adipogenic hormone for these cells (Morikawa et al., 1984), similarly to cultures incubated with 25 ng/ml hPL, cultures transfected with 0.5-5.0 μg/ml plasmid with the hPL-2 gene did not undergo adipose conversion although the secreted hormone reached a concentration of 5.0 ng/ml (Fig. 3; Table 2). For further experiments, we chose to use 1.0 μg/ml plasmid DNA (Table 2).

Secretion and adipogenic activity of hGH/hPL chimeric proteins

It is well known that hGH and hPL genes are formed by 5 exons from which exon-I encodes part of the signal peptide; they share about 95% nucleotide identity and about 85% identity in amino acid sequence (Bewley et al., 1972; Cooke et al., 1981), but hPL preparations do not have growth promoting (Aloj and Edelhoch, 1971) or adipogenic activities (Fig. 2) (Morikawa et al., 1984). Therefore, different constructs of hGH/hPL chimeric genes (Table 1) should allow the analysis and identification of hGH exons active in adipose cell differentiation. We constructed hGH/hPL chimeric molecules by homologous mutagenesis (Reséndez-Pérez and Barrera-Saldaña, 1990) as described in Materials and Methods (Fig. 4). Then, the 60% confluent 3T3-F442A cells cultured in non-adipogenic medium were transiently transfected by lipofection with the chimeric genes, and further incubated in serum-free medium for 72 hours to

![Fig. 1. Schematic representation to illustrate the culture conditions used in experiments.](image-url)
confirm that hGH/hPL chimeric proteins were synthesized and secreted by transfected cells. Conditioned serum-free medium contained levels of 5-30 ng/ml of chimeric proteins as determined by RIA for both hGH and hPL (Table 2). Thus, adipocyte differentiation that would be observed in transfected cell cultures should be due to synthesis and secretion of active protein, leading to an autocrine/paracrine induction of preadipocytes.

As demonstrated in Table 2, cells transfected with constructs having hGH exons III, IV and V (GH(I,II,III,V)-PL3(I,II)) and GH(I,II,IV,V)-PL3(I,II)), secreted between 9 and 13.0 ng/ml of protein and underwent differentiation into adipocytes at levels similar to those found in cultures transfected with the hGH-N gene (GH-N) or incubated with 25 ng/ml of RhGH. Likewise, cells transfected with chimeric genes having hPL exon IV (GH(I,II,III,V)-PL4(V)), exon V (GH(I,II,III,IV,V)-PL5(V)) or both exons (GH(I,II,III,IV,V)-PL4(V)), secreted between 9 to 15 ng/ml of protein and also underwent adipocyte differentiation to

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Nomenclature</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>pNUTGH</td>
<td>GH-N</td>
<td>hGH normal (N) gene</td>
</tr>
<tr>
<td>pNUThPL-2</td>
<td>PL-2</td>
<td>hPL-2 gene (Barrera-Saldaña et al., 1983)</td>
</tr>
<tr>
<td>pNUTh[GH]-3:m I,II</td>
<td>GH(I,II,III,V)-PL2(m,II)</td>
<td>Engineered hPL-3 exons I and II, with a donor splice site point mutation at the second intron (Reséndez-Pérez et al., 1990) were combined with hGH-N exons III to V.</td>
</tr>
<tr>
<td>pNUTh[GH]-3:1,II</td>
<td>GH(I,II,III,V)-PL3(I,II)</td>
<td>Contain hPL exons I and II from hPL-2 or hPL-3, and hGH-N exons III, IV and V.</td>
</tr>
<tr>
<td>or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-2:1,II</td>
<td>GH(I,II,III,V)-PL2(I,II)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:IV</td>
<td>GH(I,II,III,V)-PL3(IV)</td>
<td>Combines hPL-3 exon IV with hGH-N exons I, II, III and V.</td>
</tr>
<tr>
<td>pNUTh[GH]-3:V</td>
<td>GH(I,II,III,V)-PL3(V)</td>
<td>Combination of hPL-3 exon V with hGH-N exons I to IV.</td>
</tr>
<tr>
<td>pNUTh[GH]-2:IV,V</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td>Contains hPL-2 exons IV and V, and hGH-N exons I to III.</td>
</tr>
<tr>
<td>pNUTh[GH]-2:1,II</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:IV</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
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<tr>
<td>or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-2:1,II</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:IV</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:1,II</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:II</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
<td></td>
</tr>
<tr>
<td>pNUTh[GH]-3:IV</td>
<td>GH(I,II,III,V)-PL3(IV,V)</td>
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</tbody>
</table>

Table 1. Nomenclature and characteristics of hGH/hPL chimeric constructs

Table shows the nomenclature given to the constructs. Left column summarizes the characteristics of the hGH/hPL chimeric genes. The hPL-2 and hPL-3 genes encode identical mature hormones (Barrera-Saldaña et al., 1983). It should be noted that exon I only encodes the signal peptide in both hPL and hGH genes. Mutation in the GH(I,II,III,V)-PL2(m,II) chimeric gene is similar to that found in the hPl-1 pseudogene (Reséndez-Pérez et al., 1990); thus translation gives rise to a truncated protein.
The hPL-2 and hPL-3 genes used to construct the proteins secreted into culture medium were quantified by RIA (see Materials and Methods). The hPL-1 gene (Reséndez-Pérez et al., 1990) (Table 2). All identical mature hormones (Barrera-Saldaña et al., 1983). The results correspond to a representative experiment (average ± s.d.) from four different duplicated experiments. *N.D.: not detected.

The 3T3-F442A preadipocytes were transfected as described (see Materials and Methods). After seven days, cultures were extracted and adipose conversion levels were determined assayng GDPH activity. Recombinant proteins secreted into culture medium were quantified by RIAs (see Materials and Methods). The hPL-2 and hPL-3 genes used to construct the GH(III,IV)-PL(III,IV) and GH(III,IV)-PL(II,II) chimeric proteins encode identical mature hormones (Barrera-Saldaña et al., 1983). The results correspond to a representative experiment (average ± s.d.) from four different duplicated experiments. *N.D.: not detected.

levels similar to cells incubated with the hGH-N gene or 25 ng/ml of RhGH (Table 2).

In contrast, adipogenic activity did not result from chimeric proteins which had only both hGH exons I and II, only hGH exon III or only hGH exon IV (Table 2). Also, no adipogenic activity was found when cultures were transfected with GH(III,IV)-PL(II,II), a plasmid that encodes a truncated mRNA due to a mutation at the donor splice site of intron 2 of the hGH-1 gene (Reséndez-Pérez et al., 1990) (Table 2). All these chimeric proteins were not adipogenic despite the fact that transfected cells secreted similar levels of proteins to those secreted by cultures transfected with the adipogenic chimeric genes (Table 2). The concentration of secreted chimeric proteins in the culture was about 150-fold higher than the minimum concentration of hGH in medium required to promote about 50% adipose conversion. The results demonstrate that constructs showing adipogenic activity encoded hGH exon III together with hGH exon II or with hGH exon IV. These results suggest that adipogenic activity of human growth hormone depends upon the presence of functional hGH binding site-1, located in exon III, together with some sequences of binding site-2, those located either in exon II or in exon IV.

**DISCUSSION**

Major evidence leading to the understanding of hGH interaction with its receptors has been obtained by mutagenesis and structural analysis (Cunningham et al., 1989, 1992; De Vos et al., 1992; Lowman et al., 1991). However, data derived from crystallographic and receptor-binding studies do not necessarily reflect the biological activity of the mutant proteins (Helman et al., 1997; Behncken et al., 1997; Vashdi-Elberg et al., 1995). To have a better understanding of hGH biological activity, we studied the domains of hGH necessary for adipose conversion through generation of hGH/hPL chimeric genes by homologous mutagenesis (Reséndez-Pérez and Barrera-Saldaña, 1990; Cunningham et al., 1989, 1992; Lowman et al., 1991), and tested the adipogenic activity of the expressed proteins in transiently transfected 3T3-F442A cell cultures. This approach has four main advantages: (1) homologous DNA mutagenesis produces chimeric proteins with minimal changes in secondary and tertiary structure (Cunningham et al., 1989). (2) Chimeric proteins are assayed in a cell system highly sensitive to hGH, but not responsive to hPL (Morikawa et al., 1984). (3) Purification of chimeric molecules is obviated since bioassay is based on autocrine/paracrine induction of transfected cells. (4) This autocrine/paracrine biological model seems to have great potential for a simple analysis of modified genes and expression of their products through their secretion and stimulation of biological functions in transfected suitable cell types.

Studies of mutagenesis have suggested that hGH biological activity requires the sequential interaction and dimerization of hGH molecules with two cellular receptors (Cunningham et al., 1991; De Vos et al., 1992). Interaction with a first receptor is mediated through hGH binding site-1, formed by three discontinuous regions comprising the loop between hGH residues 54-74 (exon III), the COOH terminus from helix 4 (exon V) and, to a lesser extent, the NH2-terminal region from exon II.

**Table 2. Adipogenic activity and secretion of hGH/hPL chimeric proteins**

<table>
<thead>
<tr>
<th>Addition</th>
<th>GDPH activity (nmoles NADH/mg prot/min)</th>
<th>Secreted protein (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td>hGH</td>
</tr>
<tr>
<td>Non-adipogenic medium</td>
<td>29.4±2.0</td>
<td>28.3±1.9 N.D.*</td>
</tr>
<tr>
<td>25 ng/ml hGH</td>
<td>592.0±86.0</td>
<td>15.5±0.7 N.D.*</td>
</tr>
</tbody>
</table>

**Table 3. Changes introduced in hGH sequence after homologous mutagenesis**

<table>
<thead>
<tr>
<th>hGH/hPL chimeric genes</th>
<th>Change in amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH(III,IV)-PL(II,II)</td>
<td>Q84E, Q91R, V69M, S100N, G104D, A105T, N109D, V110D, D112H</td>
</tr>
<tr>
<td>GH(III,IV)-PL(IV)</td>
<td>Q84E, Q91R, V69M, S100N, G104D, A105T, N109D, V110D, D112H</td>
</tr>
</tbody>
</table>

Table summarizes the changes in amino acid sequence introduced into the hGH/hPL chimeric proteins after homologous mutagenesis of hGH. We only show the mutations for those chimeric proteins analyzed in this paper. Mutations previously reported as binding determinants during interaction of hGH with receptors (Cunningham et al., 1991; Lowman et al., 1991) are highlighted and marked with an asterisk. Mutations that modify hGH binding to receptor (Cunningham et al., 1991; Lowman et al., 1991) are shown in underlined italics (see Discussion).
helix 1 (exon II) (Cunningham et al., 1989). Growth hormone binds to a second receptor, through its binding site-2 mainly constituted by residues 1-8 (exon II) and 109-128 (exon IV) (Cunningham et al., 1991). Mutations disrupting the integrity of the binding site-1 have led to a null interaction of hGH with receptor (Cunningham et al., 1989); and mutations in residues 1-8 and on 116 of binding site-2 reduced receptor dimerization (Silva et al., 1993).

The functional epitope of hGH comprised in the binding site-1 seems to be configured by residues K41, L45, P61 and R64 encoded by exon III, and K172, T175, F176 and R178 encoded by exon V (Cunningham and Wells, 1993). It was previously described that the I179M single substitution in hGH reduced 5-fold its binding to the first receptor (Lowman et al., 1991). Our results showed that the chimeric proteins not altered in the functional epitope in binding site-1 were adipogenic. Thus, the GH(I,II,III)-PL(V) chimeric protein was highly adipogenic in spite of the absence of hGH exons IV and V, that has 14 amino acid differences from intact hGH (Table 3). These two chimeric proteins do not have changes at the functional epitope of binding site-1; in both cases substitution of hGH exon V with hPL exon V conserved the specific residues from functional epitope K172, T175, F176 and R178 encoded by hGH exon V. The chimeric proteins that

...
have hPL exon III, GH(I,II)-PL(III,IV,V) and GH(IV)-PL(I,II,III,V) did not show adipogenic activity. We conclude that adipogenic activity seems to depend upon the preservation of the hGH functional epitope in binding site-1 encoded in part by exon III.

However, the GH(III)-PL(I,II,IV,V) chimeric protein, that has the hGH functional epitope in binding site-1, did not promote adipose conversion. The lack of adipogenic activity of this chimeric protein could be due to the modification of binding site-2 by hPL exons II and IV, having the substitutions F1V, I4V and N12H in exon II and N109D, V110D and D112H in exon IV (Table 3). It was previously shown that these mutations reduced in about 2- to 4-fold the binding to hGHbp (Cunningham et al., 1989; Lowman et al., 1991) and about 5.5-fold receptor dimerization (Cunningham et al., 1991). It has been suggested that the integrity of hGH binding site-2 seems to be required for full biological activity of hGH (Cunningham et al., 1991; De Vos et al., 1992; Fuh et al., 1992, 1993; Ilondo et al., 1994).

Together, these results suggest that full adipogenic activity of hGH requires the presence of functional binding sites-1 and -2. However, all those chimeric proteins that were adipogenic contained the functional epitope encoded in hGH exons III and V together with only either hGH exon II or hGH exon IV. Binding site-2 is encoded by sequences found in both exons. The chimeric proteins GH(III,IV,V)-PL2(I,II) and GH(III,IV,V)-PL3(I,II) were adipogenic, although they contained the mutations F1V, I4V and N12H in binding site-2 that by themselves decrease dimerization by 5.4, 55 and 1.1-fold, respectively (Cunningham et al., 1989, 1991; Lowman et al., 1991) (Table 3). Likewise, the chimeric proteins GH(I,II,III)-PL(IV,V) and GH(I,II,III,V)-PL(IV), mutated in exon IV due to hPL exon IV replacement, but not in exon II were also adipogenic despite substitutions N109D, V110D and D112H in exon IV that by themselves reduced receptor dimerization by 5.5-fold (Cunningham et al., 1991) (Table 3). Since both exons II and IV encode sequences involved in binding site-2 conformation and since adipogenic activity is preserved by the presence of either one from hGH, we suggest that hGH binding site-2 might be composed by two functional subdomains; a NH2-terminal subdomain encoded by hGH exon II (subsite 2A), and a COOH-terminal subdomain encoded by hGH exon IV (subsite 2B) (see Fig. 5). Since reconstitution of hGH binding site-1 in hPL is not enough to promote adipogenesis, we suggest that the presence of either of the two subdomains of binding site-2, together with the functional epitope at binding site-1, is necessary for full biological activity of hGH.

The two binding sites model (Cunningham et al., 1989, 1991) proposed that hGH sequentially binds to two cellular receptors to elicit their dimerization and the possible biological activity of the hormone (Cunningham et al., 1989, 1991; De Vos et al., 1992). This model was based on mutagenic, structural and binding studies, which did not predict that hGH binding site-2 might be composed of two functional subdomains. Our results establish a link between changes in the structure of hGH and the induction of a biological process such as adipose differentiation, showing the possible existence of these subdomains in binding site-2 that would explain some earlier published results that were contradictory to the previously described two binding sites model.

Our findings may explain those apparently controversial results obtained by others for hGH mutants at binding site-2. Early in vitro experiments showed that hGH G120R mutant in exon IV did not produce receptor dimerization (Cunningham et al., 1991; Fuh et al., 1992), but more recently it was described that the same mutant dimerized porcine hGH receptors transfected into mouse L cells (Harding et al., 1996). It was also shown that the hGH G120R mutant, at about 25 to 50-fold higher concentrations than normal hGH, promoted growth in hypophysectomized rats (Mode et al., 1996), and stimulated proliferation of Nb2 cells (Dattani et al., 1995). Based on our results with the adipogenic chimeric protein GH(I,II,III)-PL(IV,V) that has the NH2-terminal subdomain (exon II) of binding site-2, but it is mutated in exon IV due to hPL exon IV replacement, that has 3 mutations in binding determinants for hGH interaction with receptors (Table 3), it is possible to explain that the biological activity of hGH G120R is due to the presence of the functional subsite-2A that we have described. This could also explain that the recently described D112G mutation in binding site-2 (hGH exon IV) showed 18 to 38% of the biological activity of native hGH as determined by induction of tyrosine phosphorylation of JAK2 and STAT5, and stimulation of metabolic responses (Takahashi et al., 1997). On the other hand, the I4A mutation in exon II of hGH reduced 55-fold receptor dimerization (Cunningham et al., 1991) but transgenic mice expressing this hGH mutation showed normal body growth (Chen et al., 1994). We can conclude that, as long as one of the binding site-2 subdomains that we describe remains intact, growth hormone might be biologically active in spite of an in vitro reduced receptor dimerization. It has been reported that a reduced affinity of mutated hormones for receptors is not necessarily correlated with a reduced biological activity (Helman et al., 1997; Behncken et al., 1997; Vashdi-Elberg et al., 1995; Gertler et al., 1996). In summary, in order to have adipogenic activity, the hGH/hPL chimeric proteins should have a common structure, the combined sequences encoded by hGH exon III together with either hGH exon II or with hGH exon IV.

In this paper, we have analyzed eight different hGH/hPL chimeric proteins, but some others were not tested. Based on our results we can predict whether other hGH/hPL chimeric proteins might show adipogenic activity. The GH(I,II,III,V)-PL(III) chimeric protein should not be adipogenic because it has a disrupted binding site-1 due to hPL exon III introduction. It is known that substitution of hGH exon III with the corresponding hPL sequence introduces E56D and R64M mutations in hGH; these mutations decreased in about 30-fold hGH binding capacity to hGHbp (Cunningham et al., 1989). It was also described that replacement of hGH exon III with the rat prolactin (rPRL) exon III caused a significant loss of binding activity of the chimeric protein to somatogenic receptors (Ray et al., 1990), but biological activity was not tested. In addition, deletion of residues 62-67 from exon III decreased hGH adipogenic activity in 3T3 cells (Uchida et al., 1990). We also expect that the GH(I,III,V)-PL(II,IV) chimeric protein, like the GH(III)-PL(II,IV,V) should be inactive due to alteration of hGH binding site-2 in both exon II and exon IV subdomains. Finally, the chimeric proteins GH(V)-PL(I,II,III,IV) and GH(IV,V)-PL(I,II,III) should be non-adipogenic since they should have altered both hGH binding sites -1 and -2 due to hPL exons II and III.

In conclusion, our results demonstrate that adipogenic
activity of hGH depends upon the amino acid sequences product of exon III combined with that of either exon II or exon IV of the hGH gene. They also suggest that hGH binding site 2 seems to be composed of two structural subdomains; only either one of them in combination with binding site-1, seems to be required for hGH adipogenic activity. It would be of great interest to determine which hGH/hPL chimeric proteins would have other growth hormone activities such as insulin-like, diabeticogenic or lipolytic effects.

Since receptor binding assays do not always correlate with biological activity exerted by hormones or cytokines (De V os et al., 1992; Lowman et al., 1991), the autocrine/paracrine biological model using transiently transfected cell types seems to be valuable for analysis of modified genes or gene constructs for production of peptide analogs with biological therapeutic activities. This approach comprises the stimulation of specific cellular responses by the secreted products encoded by the transfected gene in the transfected specific cell types.

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