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AN ACTIVE SITE OF GROWTH HORMONE FOR ELICITING THE DIFFERENTIATION OF PREADIPOSE 3T3-F442A CELLS TO ADIPOSE CELLS

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SUMMARY : In order to elucidate the active sites of growth hormone for eliciting the differentiation of preadipose 3T3-F442A cells to adipocytes, four artificial mutant variants of human growth hormone(hGH) modified in the loop region of amino acid residues 54-74 were prepared in *Escherichia coli* by site-directed mutagenesis. Although the P59A (replacement of Pro59 with Ala) variant retained almost the same biological- and receptor binding-activity as hGH, the P61A (replacement of Pro61 with Ala) and the P59A-P61A (replacement of both Pro59 and Pro61 with Ala) both exhibited about half the activity, and the Δ(62-67) variant (deletion of the residues 62-67) exhibited only about 0.1% the activity of those of intact hGH. The results suggest that Pro61 may be involved in formation of the active conformation of hGH, but Pro59 may not, and that the amino acid residues around 62-67 may be critical for the specific biological features of hGH.

Growth hormone (GH), a single polypeptide of about 190 amino acid residues (Mr 22 kDa) needed for somatic growth, exhibits a wide variety of actions *in vivo* and *in vitro*, including cell proliferation, cell differentiation, and regulation of metabolism [1]. Although a lot of studies have been reported on the structure-function relationship of GH [2-6], it is not fully known whether the active site(s) of GH are localized in a specific region of the GH molecule or if retention of most of the conformation of GH is

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required for biological activity. It is also unknown whether different active sites are responsible for different biological responses in the various target cells or if common specified sites or/and conformations of the GH molecule produce multiple biological responses.

We have prepared various human growth hormone (hGH) variants by site-directed mutagenesis and studied their structure-function relationships using mouse preadipose 3T3-F442A cells [7,8], since GH acts directly on these cells and specifically promotes their differentiation into adipose cells in a dose dependent manner [9,10]. The cells possess somatogenic GH receptors [11,12] and are a very sensitive *in vitro* biological assay system which responds to GH. Meanwhile, it has been reported that in a three-dimensional model of hGH derived from a folding model of porcine GH determined from crystal x-ray diffraction study [13], four antiparallel α-helices connected by loops are tightly associated by hydrophobic forces [7] (Fig.1). Our previous report showed that the retention of the hGH bundle structure consisting of four α-helices appears to be essential for activity, although the loop region of residues 32-46 is not [7]. In the present report, we have investigated the

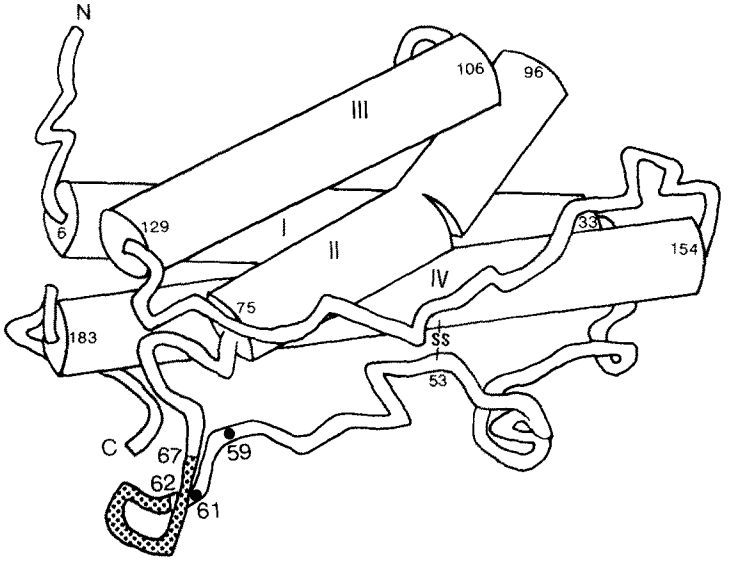

Fig. 1. Folding model of hGH based on the structure of porcine GH [13]. Cylinder represents α-helical structure. The residues are numbered according to the hGH sequence.

involvement of the long loop between Cys53 and helix II (residues 54-74) in biological activity. Two types of variants were constructed (i) by replacement of Pro59 or/and Pro61 with Ala, because Pro is a residue that fixes the polypeptide backbone, and (ii) by deletion of amino acid residues 62-67, because this region seems to project in the three-dimensional model [7]. We were able to show that the residues around 62-67 may be important as an active site of GH.

MATERIALS AND METHODS

Reagents: Various restriction enzymes, Klenow fragment of DNA polymerase, T4 DNA ligase and polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan).

Construction of Mutant Genes: The mutant genes for hGH were constructed by *in vitro* mutagenesis, using single stranded M13 phage DNA, essentially as described previously by Nishikawa *et al.* [7]. The *HpaI-SalI* DNA fragment containing the hGH gene was obtained from pGH-L9 [14] and was inserted between the *SmaI* and *SalI* sites of M13mp19 replicative-form DNA. The following deoxyoligonucleotides for the generation of mutations were synthesized by the phosphoramidite method on an automatic DNA synthesizer (Applied Biosystems, model 380A): d(GAAAGTATCGCGACCCCT) for P59A (Pro59→Ala) mutant; d(ATCCCGACCGCTTCTAAC) for P61A (Pro61→Ala) mutant; d(GAAAGTATCGCGACCGCTTCTAAC) for P59A-P61A (both Pro59 and Pro61→Ala) mutant; d(ATCCCGACCCCTCAGCAGAAATCG) for Δ(62-67) (deletion from residues 62 to 67) mutant (underlined portions indicate the changed codons).

Recombinant M13mp19 single-stranded DNA was annealed to a mutagenic oligonucleotide, and converted to double-stranded DNA by incubation with Klenow fragment and deoxynucleoside triphosphates. The resultant reaction mixture was used directly to transfect competent JM109 cells. Positive plaques were selected by plaque hybridization using [^{32}P]-labeled versions of the deoxyoligonucleotides that were used for mutation. The phage DNA was recovered by the usual method and the nucleotide sequences of the mutant genes were confirmed by Sanger's dideoxy method [15]. Each mutant gene was reinserted into the expression vector, pGH-L9 [14], after removal of the original gene.

Purification of Proteins: Expression of mutant genes in *E. coli* HB101 and purification of expressed proteins have been done by the same procedures as described previously [14]. Recovered proteins were rechromatographed by Mono Q (HR 5/5) FPLC (Pharmacia) and 10-15 mg of purified hGH variants were obtained from 2 l of culture broth. The purity of the recovered fractions was analyzed by SDS-PAGE [16] and protein concentrations were determined by Lowry's method.

Adipose Conversion Assay: Stocks of 3T3-F442A cells [17] (kindly provided by Dr. H. Green of Harvard Medical School) were maintained in the Dulbecco-Vogt modification of Eagle's medium (DMEM) supplemented with 10% calf serum. For experiments, exponentially growing cells were trypsinized and seeded into 35-mm tissue culture dishes at about 1 x 10^4 cells in growth medium: DMEM supplemented with 3% cat serum and 2% calf serum. After 4 days, when the cells became subconfluent, the medium was replaced with conversion medium: DMEM supplemented with 1.5% cat serum, 1% calf serum, bovine insulin (5 μg/ml), human transferrin (5μg/ml), triiodothyronine (2 x 10^{-9} M), biotin (10^{-6} M), 2-mercaptoethanol (40 μM), and mouse epidermal growth factor (30 ng/ml), and then hGH variant proteins were added to the medium. Cells were cultured about

10 days to undergo adipose conversion without further change of medium, and then harvested in 25 mM Tris-HCl with 1 mM EDTA (pH 7.5), sonically disrupted, and supernatants were obtained after centrifugation at 12000 x g. The activity of glycerophosphate dehydrogenase (GPDH) in the supernatant was measured as the oxidation of NADH at 340 nm [18]. One unit of GPDH activity is defined as the oxidation of 1 nmol of NADH per minute.

Radioreceptor Assay: Intact hGH was iodinated to a specific activity of 2.59-4.63 MBq/μg using the chloramine-T method [19]. Confluent monolayer cultures of 3T3-F442A cells in growth medium were rinsed three times with Hanks' balanced salt solution containing 0.1% (W/V) bovine serum albumin and 10 mM HEPES, and incubated with [^{125}I]hGH (10^{-10} M) and unlabeled hGH variant proteins in the same buffer for 90 min at 23 ℃. The binding reaction was terminated by aspirating the buffer from the culture dish, and the cell layers were washed three times with the same buffer. The cells were harvested and the cell-associated radioactivity was determined with an autogamma counter.

RESULTS AND DISCUSSION

The biological activity of hGH variants on the adipose conversion of 3T3-F442A cells has been quantified by changes in GPDH activity, a marker enzyme of adipocyte differentiation [18]. As shown in Fig. 2, the P59A variant is nearly as active as intact hGH in adipogenic activity because both dose response curves are virtually the same. On the other hand, P61A and P59A-P61A variants exhibited about 40 and 50% of the activity of intact hGH, respectively, when compared to the hormone concentration that is 50% effective (ED_{50}). The Δ(62-67) variant showed a drastic loss of activity. The activity calculated from putative dose response curve is about 600 times less than that of hGH (Table 1).

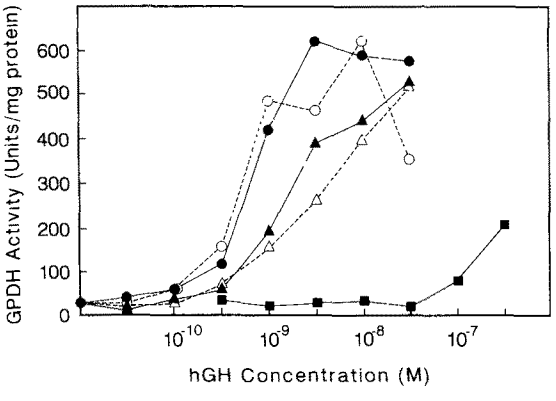

Fig. 2. Adipose conversion assay of hGH and hGH variants.
Adipogenic activities of hGH (●), P59A variant (○), P61A variant (▲), P59A-P61A variant (△) and Δ(62-67) variant (■) were measured as described in Materials and Methods. Each point indicates the average of duplicate dishes.

Table 1. Comparison of biological activity and receptor-binding activity of hGH variants

hGH Variants	Relative Biological Activity* (Adipose conversion assay) (%)	Relative Receptor-binding Activity** (Radioreceptor assay) (%)
hGH	100	100
P59A	102	138
P61A	46	67
P59A-P61A	39	36
Δ (62-67)	(0.0016)	0.0008

All values are the mean of two experiments.
* Relative adipogenic activity is calculated by comparison with ED_{50}. The value in parentheses was obtained from the putative dose-response curve.
**Relative receptor-binding activity is calculated by comparison with IC_{50}.

We then measured the receptor binding activity of hGH variants by specific inhibition of [^{125}I]hGH binding to 3T3-F442A cells. As shown in Fig. 3, the replacement of Pro59 with Ala caused no significant change in the displacement curve from that of hGH, whereas P61A and P59A-P61A showed a significant loss of binding activity. The Δ (62-67) variant again showed considerable loss of binding activity, and retained only 0.1% or less of that of hGH when the binding activity is calculated from the hormone concentration needed for 50% inhibition (IC_{50}) (Table 1).

In the three-dimensional model of hGH (Fig.1), modifications introduced in the loop region described in this paper would not be expected to induce any

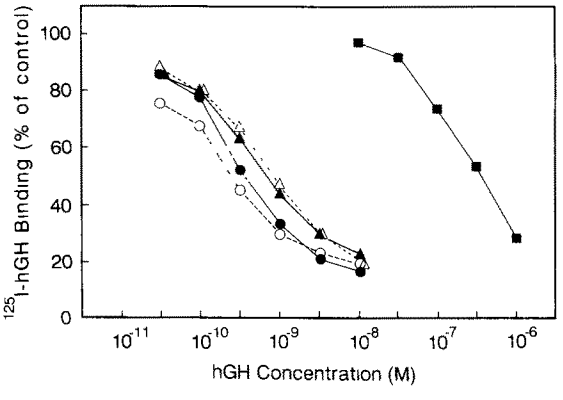

Fig. 3. Radioreceptor assay of hGH and hGH variants.
Radioreceptor assay of hGH (●), P59A variant (○), P61A variant (▲), P59A-P61A variant (△) and Δ (62-67) variant (■) was conducted as described in Materials and Methods. Each point indicates the average of triplicate dishes.

structural changes in the bundle structure, but may cause small changes in structural features, including the spatial arrangement of the microenvironment in the loop region. In fact, the α-helical contents are similar between the intact hGH and hGH variants as judged from far-UV CD spectra (data not shown). Nevertheless, the P61A and P59A-P61A variants lost about half their activity, indicating that the replacement of Pro61 with Ala seems to disrupt the most appropriate local conformation of hGH required for biological activity. The finding that the Δ (62-67) variant showed considerably loss of biological activity (Table 1) strongly suggests that an active site is located within or close to residues 62-67.

The good correlation between receptor binding and adipogenic activity in hGH variants (Table 1) suggests that the reduction of biological activities seen in P61A, P59A-P61A and Δ (62-67) variants seems to be mainly caused by a reduction in receptor binding. Thus, the region around 62-67, including Pro61, may have a crucial role in the biological activity of hGH by interacting with the GH receptor. This is the first report to identify a specific region of the hGH molecule which is essential for adipogenic activity. Recently, Cunningham *et al.* have defined three binding sites in hGH which contribute to binding to the cloned GH receptor of human liver: the NH_2-terminal region of helix I; the loop region between residues 54 and 74; and the COOH-terminal region of helix IV [5]. Further, it has been demonstrated that in the loop region of residues 54-74, a single Ala substitution which caused greater than a four-fold reduction in binding activity for the human GH receptor was observed at positions 54, 56, 58, 64 and 68 [6]. Replacement of exon 3 of hGH with that of rat prolactin caused a significant loss of binding to the somatogenic GH receptor in rabbit liver [20], suggesting that the exon 3-encoded region (residues 32-71) appears to be important for binding activity. These taken together with our reports strongly suggest that the region around residues 62-67 of hGH may be common requirements for any GH receptor-binding.

In conclusion, from our present work, it is revealed that certain structural features preserved by residues 62-67 of hGH are critical for GH receptor recognition and thereby this region may be regarded as an active site

of GH action. The appropriate conformational features of this region may be characterized by the existence of Pro61.

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