The Binding between the Stem Regions of Human Growth Hormone (GH) Receptor Compensates for the Weaker Site 1 Binding of 20-kDa Human GH (hGH) than That of 22-kDa hGH*

Bunkichi Tsunekawa‡, Mitsufumi Wada‡§, Miwa Ikeda‡, Shinichi Banba†, Hironori Kamachi‡, Eishi Tanaka†, and Masaru Honjo‡

From the ‡Pharmaceuticals Section, Life Sciences Laboratory and the ¶Computer Science Department, Material Science Laboratory, Mitsui Chemicals, Inc., 1144 Togo, Mobara-shi, Chiba 297-0017, Japan

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Despite the lower site 1 affinity of the 20-kDa human growth hormone (20K-hGH) for the hGH receptor (hGHR), 20K-hGH has the same hGHR-mediated activity as 22-kDa human GH (22K-hGH) at low hGH concentration and even higher activity at high hGH concentration. This study was performed to elucidate the reason why 20K-hGH can activate hGHR to the same level as 22K-hGH. To answer the question, we hypothesized that the binding between the stem regions of hGHR could compensate for the weaker site 1 binding of 20K-hGH than that of 22K-hGH in the sequential binding with hGHR. To demonstrate it, we prepared 15 types of alanine-substituted hGHR gene at the stem region and stably transfected them into Ba/F3 cells. Using these cells, we measured and compared the cell proliferation activities between 20K- and 22K-hGH. As a result, the activity of 20K-hGH was markedly reduced than that of 22K-hGH in three types of mutant hGHR (T147A, H150A, and Y200A). Regarding these mutations, the dissociation constant of hGH at the first and second step (KD1 and KD2) in the sequential binding with two hGHRs was predicted based on the mathematical cell proliferation model and computational simulation. Consequently, it was revealed that the reduction of the activity in 20K-hGH was attributed to the change of not KD1 but KD2. In conclusion, these findings support our hypothesis, which can account for the same potencies for activating hGHR between 20K- and 22K-hGH, although the site 1 affinity of 20K-hGH is lower than that of 22K-hGH.

The 20-kilodalton (kDa) human GH (20K-hGH) is known to be naturally secreted from the pituitary gland besides 22-kDa human GH (22K-hGH), which is a major component composed of 191 amino acids (1, 2). The 20K-hGH is encoded by the same human GH isoforms by theoretically simulating the cell proliferation curve, and it was highly speculated that alanine substitution at siteBP could mainly affect the affinity of 20K-hGH at STEP2 (KD2/20K). By lacking 15 amino acids, 20K-hGH partly lost the site 1 affinity but the binding between siteBPs possibly compensates for the loss.

Experimental Procedures

Materials—Recombinant 20K-hGH with an authentic amino acid sequence was prepared as described previously (6). As for the 22K-hGH sample, a commercially supplied recombinant one with an authentic amino acid sequence (Genotropin, Amersham Pharmacia Biotech, and Upjohn AB, Sweden) was used. IL-3-depentent mouse B cell line (Baf3) was from the RIKEN Cell Bank (Baraki, Japan).

Expression Plasmid pCXN2Zb—Expression plasmid pCXN2Zb was a derivative of pCXN2 (18). Cloning sites (EcoRI, XhoI, PvuII, KpnI, EcoRV, SacI, HpaI, HindIII, and EcoRI) were added by the insertion of a DNA fragment into the EcoRI cloning site of pCXN2.
Preparation of Ba/F3 Cells Stably Expressing hGHR—Approximately $1 \times 10^5$ Ba/F3 cells were transfected with 50 $\mu$g of the pCXN2Zb vector containing the wild type or each alanine-substituted hGHR cDNA by being pulsed at 200 V, 960 microfarad in ice-cold Opti-MEM medium (Life Technologies, Inc.). To select the cells resistant to antibiotic G418, the first selection was performed in selection medium A (RPMI 1640 medium containing 10% fetal calf serum, 50 $\mu$g/ml streptomycin, 1 mg/ml G418, and 1 mg/ml mouse IL-3). The second selection was carried out in the selection medium B (RPMI 1640 medium containing 10% fetal calf serum, 50 $\mu$g/ml streptomycin, 4 mg/ml streptomycin, 1 mg/ml G418, and 1 mg/ml mouse IL-3). The selection of Ba/F3 expressing the hGHR proliferate in response to hGH in a dose-dependent manner and are a useful and convenient tool for measuring cell proliferation activity. The expression level of the wild type or each alanine-substituted hGHR cDNA was stably transfected into the IL-3-dependent mouse pro B cell line (Ba/F3) by the electroporation method, according to the manufacturer’s protocol, and absorbance at 450 nm (reference wavelength: 595 nm) was measured.

Sequential Binding Model—Human GH sequentially binds to hGHR resulting in the formation of a dimer consisting of one molecule of hormone and two molecules of receptor (12–14). Uchida et al. (9) studied the sequential binding of hGH with hGHR on the cell surface of Ba/F3 expressing hGH and calculated the 1:2 complex concentration ([hGH:(hGHBP)2]) as functions of STEP1 and STEP2 affinity (KD1 and KD2), and [hGH:(hGHBP)2] can be represented as follows.

$$[\text{hGH:(hGHBP)2}] = f(\text{KD1, KD2})$$  \hspace{1cm} (Eq. 1)

Because we are basically using the same Ba/F3 cells, expression plasmid, and cell proliferation assay method as Uchida et al. (9), we adopted the same definition, assumptions, and prerequisites of the sequential binding model to estimate the hGH 1:2 complex concentration ([hGH:(hGHBP)2]) on the cell surface of Ba/F3 expressing hGH.

Cell Proportion Model—During the logarithmic phase of cell proliferation, cell population is represented as follows.

$$dN/dt = \mu \times N; \quad N/N_0 = \exp(\mu \times t)$$  \hspace{1cm} (Eq. 2)

Here, $t$ is the period of time for cell proliferation (h), $N_0$ is the initial cell population size, $N$ is the size of the cell population after a period of growth, and $\mu$ is the specific growth rate ($h^{-1}$).

In the cell proliferation model, we made the assumptions as follows.
1) The strength of intracellular growth signal only depends on the cell surface 1:2 complex concentration ([hGH:(hGHBP)2]) and is in direct proportion to it. 2) The strength of intracellular growth signal derived from one 1:2 complex is identical among the wild type and each mutant hGHR.

Based on above assumptions, $\mu$ is represented as follows.

$$\mu = \alpha \times [\text{hGH:(hGHBP)2}] = \alpha 	imes f(\text{KD1, KD2})$$  \hspace{1cm} (Eq. 3)

$\alpha (h^{-1} \cdot M^{-1})$ is a proportionality constant. Finally, the fold induction ($N/N_0$) in the cell proliferation assay is represented as follows.

$$N/N_0 = \exp(\mu \times t) = \exp(\alpha \times f(\text{KD1, KD2}) \times t)$$  \hspace{1cm} (Eq. 4)

Here, $\alpha$ and $t$ are constants that are determined by the experimental condition.

RESULTS

Selection of Amino Acids at the siteBP Region—The crystal structure of the ligand-bound complex shows that 1:2 complex formation of 22K-hGH with the extracellular domain of hGHR (hGH-ECD) is aided by a dimerization domain in the C-terminal $\beta$ sandwich (domain 2), namely siteBP region in Fig. 1, and eight residues (Asn-143, Ser-145, Leu-146, Thr-147, His-150, Asp-152, Tyr-200, and Ser-201) are identified as being involved in this dimerization domain (13). Based on the tertiary crystal structure of the 1:2 complex between 22K-hGH and hGH-ECD registered in Protein Data Bank (PDB ID: 3HHR), in addition to above the eight amino acids we selected seven amino acids (Leu-142, Val-144, Ile-149, Gln-154, Ile-192, Val-197, and Pro-198), which were presumed to be located at the contact surface area between siteBPs. Because alanine substitution is minimally perturbing for the secondary and tertiary structure, it is generally regarded as the best means of selective removal of interactive residues involved in salt bridges and hydrogen bonds (19, 20). We constructed 15 hGHR expression plasmids with a single alanine substitution at each of the 15 amino acids.

Cell Proportion Assay—As already reported (11), Ba/F3 cells expressing hGH proliferate in response to hGH in a dose-dependent manner and are a useful and convenient tool for measuring hGH-mediated activity. The expression plasmid containing the wild type or each alanine-substituted hGHR cDNA was stably transfected into the IL-3-dependent mouse pro B cell line (Ba/F3) by the electroporation method, according to the manufacturer’s protocol, and absorbance at 450 nm (reference wavelength: 595 nm) was measured.

$\alpha (h^{-1} \cdot M^{-1})$ is a proportionality constant. Finally, the fold induction ($N/N_0$) in the cell proliferation assay is represented as follows.
but not in the second one. Each cell expressing the wild type or mutant hGHR except for mutation D152A proliferated in response to 20K- and 22K-hGH in a dose-dependent manner; however, their dose-response curves were classified into three patterns as shown in Table I. Fig. 2 shows the representative cell proliferation curve of each pattern.

In pattern I, 20K- and 22K-hGH had equal potency at lower hGH concentration (≤10–30 nM), and the difference between both hGH isoforms was detected only at high hGH concentration. The cell proliferation curve of the wild type hGHR is shown in Fig. 2 as an example of pattern I, into which mutations L142A, V144A, S145A, L146A, T147A, I192A, V197A, P198A, and S201A are classified. In pattern II, to which only mutation N143A belonged, cells proliferated even without hGH stimulation, and hGH had relatively lower fold induction than the other mutations probably because of the higher basal proliferation activity at 0 nM hGH. In pattern III, 20K-hGH had diminished activity compared with 22K-hGH. The cell proliferation curve of mutant hGHR (Y200A) represents this pattern III to which mutation T147A, I149A, and H150A belonged. In the assay of mutant T147A, H150A, and Y200A, the maximum fold induction level of 20K-hGH was decreased by 8.4, 22, and 33% relative to that of 22K-hGH, respectively, even at higher 20K-hGH concentration than 22K-hGH. Regarding the mutation I149A, the maximum fold induction level was the same between both hGHs, but EC50 of 20K-hGH was about three times larger than that of 22K-hGH.

### Computer-aided Simulation of Cell Proliferation Curve

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man GH sequentially binds to two hGHRs, resulting in the formation of a 1:2 (hGH:hGHR) complex (12–14). This sequential binding mechanism was studied by a mathematical model (21), and 1:2 complex concentration ([hGH:(hGHBP)2]) on the cell surface of Ba/F3 expressing hGHR was calculated as functions of KD1 and KD2 (9). In this report, we have developed a mathematical cell proliferation model via hGHR based on the sequential binding model of hGHR.

The binding affinity of 22K-hGH at STEP1 (KD1(22K)) for the wild type hGHR has been studied using the hGHR-ECD

### Table I

Summary of proliferation patterns of Ba/F3 cells expressing the wild type or each mutant hGHR

| Mutation | Cell proliferation pattern |
|----------|---------------------------|
| Wild type | I                         |
| L142A    | I                         |
| N143A    | II                        |
| V144A    | I                         |
| S145A    | I                         |
| L146A    | I                         |
| T147A    | III                       |
| I149A    | III                       |
| H150A    | III                       |
| D152A    | N.D.                      |
| Q154A    | I                         |
| I192A    | I                         |
| V197A    | I                         |
| P198A    | I                         |
| Y200A    | III                       |
| S201A    | I                         |

### Figures

**Fig. 2.** Representative cell proliferation curve induced by 20K- or 22K-hGH stimulation. The fold induction was calculated as absorbance in the presence of hGH divided by absorbance in the absence of hGH. **Pattern I:** cells were GH-dependent, and the activity of 20K-hGH was the same as that of 22K-hGH at low hGH concentration and higher at high hGH concentration. **Pattern II:** cells proliferated even without hGH stimulation. **Pattern III:** cells were GH-dependent and proliferated by 20K-hGH to the less extent than by 22K-hGH. Each data point represents the mean ± S.D. from triplicate wells.

**Fig. 3.** Experimental and calculative proliferation curve induced by 20K- and 22K-hGH. Fold induction (N/N0) curves were calculated using Equation 4 under "Experimental Procedures." **A:** wild type hGHR; **B:** mutation T147A; **C:** mutation I149A; **D:** mutation H150A; **E:** mutation Y200A. **T:** calculative proliferation curve; **E:** experimental data points.
by some groups, and it is considered to be 1–3 nM (9, 22). Moreover, Pearce et al. (23) mentioned in their discussion that the KD2(22K) value for the wild type hGHR was estimated to be ~0.5–5 nM. Therefore, we first adopted KD1(22K) = 2 (nM) and KD2(22K) = 2 (nM) as initial values to determine the initial value of α in the Equation 4. The calculated fold induction (N/N0) was fitted to the actual experimental values of cell proliferation assay with 22K-hGH and the wild type hGHR when the period of time for cell proliferation (t) was 20 h. Consequently, the initial value of α was calculated as 2.30 × 10^5 h^{-1}M^{-1}. Secondly, KD1(22K), KD2(22K), and α were optimized using a systematic search on a grid, by minimizing the root mean square deviations between observed data and model calculations. The grid size and range used for KD1(22K) and KD2(22K) were 0.1 and 0–150 nM, respectively, and those for α were 1.0 × 10^3 h^{-1}M^{-1} and 2.20–3.00 × 10^5 h^{-1}M^{-1}, respectively. As a result, as shown in Fig. 3A, when 5.1 nM, 2.9 nM, and 2.41 × 10^5 h^{-1}M^{-1} were adopted as KD1(22K), KD2(22K), and α, respectively, the simulated curve gave the best fit to the experimentally obtained cell proliferation curve with 22K-hGH and the wild type hGHR. Finally, KD1 and KD2 of 20K-hGH via the wild type hGHR and those of both hGH isoforms via mutant hGHR (T147A, I149A, H150A, and Y200A) in pattern III were also optimized using a systematic search on a grid. The same grid size and range were used for them. α was kept constant at 2.41 × 10^5 h^{-1}M^{-1} as in the case of 22K-hGH and the wild type hGHR during the systematic search, because the experimental condition for the cell proliferation assay was identical. The adopted KD1 and KD2 value of 20K- and 22K-hGH are summarized in Table II.

| Mutation     | KD1(20K) | KD2(20K) | R1/20K: KD1(20K)/KD1(20K-Wild Type) | R2/20K: KD2(20K)/KD2(20K-Wild Type) |
|--------------|----------|----------|-----------------------------------|-----------------------------------|
| Wild type    | 13       | 1.0      | 1.0                               | 1.0                               |
| T147A        | 28       | 10       | 2.2                               | 2.0                               |
| I149A        | 16       | 4.2      | 1.3                               | 4.2                               |
| H150A        | 30       | 122      | 2.4                               | 122                               |
| Y200A        | 40       | 68       | 3.2                               | 68                                 |

| Mutation     | KD1(22K) | KD2(22K) | R1/22K: KD1(22K)/KD1(22K-Wild Type) | R2/22K: KD2(22K)/KD2(22K-Wild Type) |
|--------------|----------|----------|-----------------------------------|-----------------------------------|
| Wild type    | 5.1      | 2.9      | 1.0                               | 1.0                               |
| T147A        | 3.0      | 5.2      | 0.59                              | 1.8                               |
| I149A        | 4.1      | 4.9      | 0.80                              | 1.7                               |
| H150A        | 3.3      | 50       | 0.65                              | 17                                |
| Y200A        | 3.4      | 15       | 0.67                              | 5.3                               |

B: KD1 and KD2 of 22K-hGH

In patients with Laron syndrome (familial GH resistance characterized by severe dwarfism and metabolic dysfunction), a point mutation in the siteBP region was identified resulting in the substitution of a highly conserved aspartate residue by histidine at position 152 (D152H) of hGHR (26). Duquesnoy et al. (26) reported that the hGH with mutation D152H displayed subnormal GH binding activity but hGH-ECD with D152H substitution was unable to dimerize. In this report, not only 22K-hGH but also 20K-hGH had no cell proliferation activity in mutation D152A nor D152H (data not shown). These results mean that Asp-152 in hGHR plays a quite important role in the binding between siteBP regions.

Especially, the mutation T147A, H150A, and Y200A of hGH resulted in a drastic decrease of cell proliferation activity of 20K-hGH compared with that of 22K-hGH. Similar results were observed also in the rat serine protease inhibitor 2.1 gene promoter activation assay in CHO-K1 cells transiently expressing each three mutants (data not shown). These data indicate that Thr-147, His-150, and Tyr-200 considerably contribute to the same hGH-mediated activity of 20K-hGH as 22K-hGH regardless of its reduced site 1 affinity. To clarify the mechanism of how Thr-147, His-150, and Tyr-200 enable 20K-hGH to form an active 1:2 complex with hGHR-ECD, Crystallographic study showed the eight residues (Asn-143, Ser-145, Leu-146, Thr-147, His-150, Asp-152, Tyr-200, and Ser-201) were involved in this domain. Furthermore, Clackson et al. (24) showed that hGHR dimerization stabilized loop structure from Val-144 to Gly-148 at siteBP region. Chen et al. (25) converted several amino acids at siteBP of rabbit GHR to alanine, aspartate, lysine, or cysteine and presented that residues Ser-145, His-150, Asp-152, Tyr-200, and Ser-201 were required for effective signal transduction through the dimerization domain. These previous studies are well consistent with our finding except for Ile-149, because the involvement of Ile-149 in siteBP interaction has been demonstrated for the first time in this study.

DISCUSSION

In this study, we have revealed that alanine substitution at Thr-147, Ile-149, His-150, and Tyr-200 in the siteBP region of hGHR reduced the cell proliferation activity of 20K-hGH as compared with that of 22K-hGH. The involvement of the siteBP region in the complex formation of 22K-hGH and hGHR has been studied by several groups. DeVos et al. (13) first reported that there was a substantial contact surface between the C-terminal domains of hGHR-ECD, namely siteBP regions, when 22K-hGH formed a 1:2 complex with hGHR-ECD. Crystallographic study showed the eight residues (Asn-143, Ser-145, Leu-146, Thr-147, His-150, Asp-152, Tyr-200, and Ser-201) were involved in this domain. Furthermore, Clackson et al. (24) showed that hGHR dimerization stabilized loop structure from Val-144 to Gly-148 at siteBP region. Chen et al. (25) converted several amino acids at siteBP of rabbit GHR to alanine, aspartate, lysine, or cysteine and presented that residues Ser-145, His-150, Asp-152, Tyr-200, and Ser-201 were required for effective signal transduction through the dimerization domain. These previous studies are well consistent with our finding except for Ile-149, because the involvement of Ile-149 in siteBP interaction has been demonstrated for the first time in this study.

In patients with Laron syndrome (familial GH resistance characterized by severe dwarfism and metabolic dysfunction), a point mutation in the siteBP region was identified resulting in the substitution of a highly conserved aspartate residue by histidine at position 152 (D152H) of hGHR (26). Duquesnoy et al. (26) reported that the hGH with mutation D152H displayed subnormal GH binding activity but hGH-ECD with D152H substitution was unable to dimerize. In this report, not only 22K-hGH but also 20K-hGH had no cell proliferation activity in mutation D152A nor D152H (data not shown). These results mean that Asp-152 in hGHR plays a quite important role in the binding between siteBP regions.

Especially, the mutation T147A, H150A, and Y200A of hGH resulted in a drastic decrease of cell proliferation activity of 20K-hGH compared with that of 22K-hGH. Similar results were observed also in the rat serine protease inhibitor 2.1 gene promoter activation assay in CHO-K1 cells transiently expressing each three mutants (data not shown). These data indicate that Thr-147, His-150, and Tyr-200 considerably contribute to the same hGH-mediated activity of 20K-hGH as 22K-hGH regardless of its reduced site 1 affinity. To clarify the mechanism of how Thr-147, His-150, and Tyr-200 enable 20K-hGH to form an active 1:2 complex to the same degree as 22K-hGH, we are now under investigation of the x-ray crystal structure of the complex of 20K-hGH and hGHR-ECD.

To elucidate the binding affinity at STEP1 and STEP2 resulting from alanine substitution, we estimated the dissociation constant KD1 and KD2 of 20K- and 22K-hGH by modeling the cell proliferation curve mathematically. By fitting the calculated proliferation curve to the experimental data of the wild type hGHR, KD1(20K) and KD2(22K) were predicted to be 13 nM.
and 5.1 nm, respectively, and the binding affinity of 20K-hGH at site 1 was weaker than that of 22K-hGH. These estimates are reasonable compared with the results experimentally obtained by biosensor analysis using hGHR-ECD, where KD1(20K) (16 nM) is larger than KD1(22K) (2 nM) (9).

Concerning the mutation T147A, H150A, and Y200A, KD2(20K) increased 10-, 122-, and 68-fold, respectively, compared with KD2(22K) (1.8-, 17-, and 5.3-fold). Generally alanine substitution is minimally perturbing for the secondary and tertiary structure, and three alanine mutations are located only at the siteBP region of hGHR. Therefore, mutations T147A, H150A, and Y200A are considered to have no direct influence on the contact surface structure between the site 2 region on hGH and the second hGHR in sequential binding, that is, the binding affinity KDsite2(20K). However, Rowlinson (27) reported that GH bioactivity was in good concordance with the change of the binding affinity KD2(20K). It is predicted that an ~30-fold reduction in site 1 affinity should yield about 30-fold higher EC50 for cell proliferation, which means the experimental data of Pearce et al. (23) could not be simulated by our model. Unfortunately, at present we cannot explain such disagreement, and some modifications might be necessary for more accurate simulation by adding some new parameters to the sequential binding model or the cell proliferation model or by altering some prerequisites.

In conclusion, we have shown that some alanine substitutions at the siteBP region of hGHR caused the markedly decreased activity of 20K-hGH compared with 22K-hGH. This means the siteBP region is involved in 1:2 complex formation in a different manner between 20K- and 22K-hGH, and the binding between the siteBP regions compensates for the weaker site 1 binding of 20K-hGH than that of 22K-hGH.

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