Analysis of binding properties between 20 kDa human growth hormone (hGH) and hGH receptor (hGHR): the binding affinity for hGHR extracellular domain and mode of receptor dimerization

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ABSTRACT

It has recently been shown that 20 kDa human growth hormone (hGH) forms the 1:2 hGH:hGH receptor (hGHR) complex and expresses full agonistic activity, although it hardly forms the 1:1 GH:GHR complex as compared with 22 kDa hGH. To clarify this mechanism, we analyzed the mode of receptor dimerization of 20 kDa hGH using the intact form and mutants. Complex formation analysis between hGHR extracellular domain (hGHBP) and either site1 mutant (K157A) or site2 mutant (G105R) by gel-filtration showed that the site1 mutant apparently formed no 1:1 complex and that the site2 mutant formed only the 1:1 complex. Cell proliferation analysis revealed that the activity curve (vs ligand concentration) of 20 kDa hGH showed a bell-shaped pattern. This indicates that the receptor dimerization of 20 kDa hGH proceeds in a sequential manner. Based on this sequential binding we have produced a mathematical model for receptor dimerization as a function of [hGH], [hGHBP], K_d values for the first

hGHBP binding (K_{d1}) and the second hGHBP binding (K_{d2}). The result of 20 kDa hGH binding to (S201C) hGHBP immobilized on biosensor tip showed that the K_{d1} value was 1.6×10^{-8} M. Adopting this value as a constant in the function described above, we have obtained calculative hGHR dimerization curves vs hGH concentration. Since the K_{d2} value could not be experimentally determined, the curves were simulatively obtained with varied K_{d2} values. The simulated curve pattern coincided with the experimental result of the cell proliferation in Ba/F3-hGHR when the value 2.5×10^{-10} M was adopted as K_{d2} . In conclusion, although the affinity of 20 kDa hGH for the first hGHR binding is reduced to one-tenth, that for the second binding is increased ten-fold in comparison with those of 22 kDa hGH, indicating that 20 kDa hGH can be an effective hGH isoform in the presence of hGHBP.

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INTRODUCTION

A molecule of 22 kDa human growth hormone (hGH) which is the most abundant hGH isoform, representing 80–90% of pituitary hGH (Baumann 1991) contains two binding sites, site1 with high affinity and site2 with low affinity, and sequentially binds two molecules of hGH receptor (hGHR) (Cunningham *et al.* 1991). Ilondo *et al.* (1994) have theoretically demonstrated that the activity curve vs hGH concentration is a biphasic

bell-shaped pattern when the receptor binding proceeds sequentially.

As for 20 kDa hGH, the second most abundant hGH isoform, the receptor binding affinities and the mode of receptor dimerization remain unclear. This natural isoform representing 5–10% of circulating hGH (Ishikawa *et al.* 1999, Tsushima *et al.* 1999) is formed by alternative splicing of mRNA from the *GH-N* gene and consequently lacks residues 32–46 in 22 kDa hGH (Masuda *et al.* 1988). The residues 32–46 of 22 kDa hGH contain C-terminal portion of

helix1 and minihelix1 which are involved in the first hGHR binding at site1. It has been reported that the acidic residue E34 of 22 kDa hGH is also involved in calcium chelating between hGH and hGHR (Rowlinson *et al.* 1994). This structural deficiency of 20 kDa hGH implies that the isoform shows diminished binding affinity for hGHR and can be a weaker agonist as compared with 22 kDa hGH. In contrast with this structural prospect, we have recently revealed that 20 kDa hGH shows full agonistic actions mediated through hGHR (Wada *et al.* 1997, 1998) and hardly forms the 1:1 self-antagonistic complex (Wada *et al.* 1998).

In this study we attempted to clarify the mode of the receptor binding and affinities for GHR in 20 kDa hGH by a combination of experimental analyses and theoretical simulation. Here we temporally designate the first binding step 'STEP1' and the second binding step forming the 1:2 complex 'STEP2'. The affinities corresponding to these two steps are designated K_{d1} and K_{d2} .

MATERIALS AND METHODS

hGH isoforms and hGHBP

Recombinant 20 kDa hGH and its mutants were produced using the *E. coli* secretion system as previously reported (Uchida *et al.* 1997). Human GHR extracellular domain (hGHBP) and its mutants were prepared using the same secretion system. For 22 kDa hGH, Genotropin (Pharmacia-Upjohn, Stockholm, Sweden) was used.

Gel-filtration and cell proliferation

Formation of hGH-hGHBP complex and gel-filtration analysis were carried out according to Roswall *et al.* (1996). Construction of the Ba/F3 which stably expresses hGHR and hGHR-mediated proliferation assay have been reported previously (Wada *et al.* 1998).

Biosensor analysis

Biosensor analysis was performed according to Cunningham and Wells (1993) in principle. As a biosensor, BIAcore (Amersham Pharmacia Biotech, Uppsala, Sweden) was used. On-rate (k_a) and off-rate (k_d) profiles were measured at 25 °C in HBS buffer (10 mM HEPES, pH 7.4 containing 150 mM NaCl, 3.4 mM EDTA and 0.005% Surfactant P20). The (S201C) hGHBP was immobilized at a level over 1000 RU (1.0 ng/mm²) on the biosensor via thiol residue of 201C. Association rates were

measured by injecting hormone solutions (200 nM, 100 nM and 50 nM of each hGH isoform in HBS buffer). Dissociation rates were measured by substituting the hormone-free HBS buffer. Flow rates of both buffer were 20 µl/min. The matrix was regenerated by washing for 20 s with 4.5 M MgCl₂. Both association and dissociation rate constants were determined using the Pharmacia Evaluation software to solve the rate equations (Karlsson *et al.* 1991).

Sequential binding model

Definition of the model

When the binding of hGH to hGHBP proceeds in a sequential manner, the binding can be expressed as follows:

where hGHBP is the extracellular domain of hGHR and k_a and k_d represent rate constants for association and dissociation, respectively. The equilibrium dissociation constants are expressed by $K_{d1} = k_{d1}/k_{a1}$ and $K_{d2} = k_{d2}/k_{a2}$.

Assumptions of the model

This model is based on the following assumptions:

(1) The biological effect of hGH is represented by concentration of hGH:(hGHBP)₂ that is a function of [hGH₀], [hGHBP₀], K_{d1} and K_{d2} .

(2) The hGHR is localized only on the cell surface.

(3) The concentration of hGH in the cell surface proximal space (space A) is in equilibrium with that in the distal space (space B). The thickness of space A (300 Å) is estimated to be approximately twice as long as the sum of the length for the hGHR extracellular domain (75 Å) and the diameter of the 22 kDa hGH molecule (80 Å). The number of expressed hGHRs on the cellular surface of Ba/F3-hGHR was 3800 per cell, which was obtained by Scatchard analysis. Using this value, the concentration of hGHR (hGHBP₀) in space A was calculated to be 0.67 µM.

In the assumption, balance equations of hGH and hGHBP are represented as follows:

$$\begin{aligned} \text{hGH: } (n+1) &= (n+1) \\ &[\text{hGH}_0] - [\text{hGH:hGHBP}] - [\text{hGH:(hGHBP)}_2] \\ \text{hGHBP: } [\text{hGHBP}] &= [\text{hGHBP}_0] - \\ &[\text{hGH:hGHBP}] - 2[\text{hGH:(hGHBP)}_2] \end{aligned}$$

where n , hGH_0 and hGHBP_0 represent volume fraction of space B/space A, initially presented free hGH and hGHBP respectively. The n value (space B/space A) was estimated to be 2.64×10^5 .

Computer-aided simulation of the model

In the sequential binding model, the concentration of the 1:2 complex is represented as follows:

$$[\text{hGH}:(\text{hGHBP})_2] = \frac{([\text{hGHBP}_0] - [\text{hGH}:\text{hGHBP}]) \cdot [\text{hGH}:\text{hGHBP}]}{K_{d2} + 2[\text{hGH}:\text{hGHBP}]} \quad (1)$$

where $[\text{hGH}:\text{hGHBP}]$ (1:1 complex) is given as the solution (X) of the following equation:

$$\left(\frac{4K_{d1}}{K_{d2}} - \frac{1}{n+1} \right) X^3 + \left(4K_{d1} + 2[\text{hGH}_0] - \frac{K_{d2}}{n+1} \right) X^2 + \left(K_{d1} \cdot K_{d2} - 2[\text{hGH}_0][\text{hGHBP}_0] + K_{d2}[\text{hGH}_0] + \frac{K_{d2}[\text{hGHBP}_0] + [\text{hGHBP}_0]^2}{n+1} \right) X - K_{d2}[\text{hGHBP}_0] = 0 \quad (2)$$

Titration calorimetry

Aliquot (1.6 ml) of 15 μM hGHBP solution was titrated with 10 μl injections of 50 μM of each hGH isoform every 4 min on Microcal Omega calorimeter. Enthalpy was calculated using the Microcal Origin program. Statistical comparison was made using Student's t -test.

Relation between binding affinity and enthalpy

The entire process of receptor dimerization and the change of Gibbs free binding energy are represented as follows:

where k_a and k_d represent association constant and dissociation constants respectively. The value K_d can be obtained by the values of ΔH (enthalpy change) and ΔS (entropy change).

RESULTS

Gel-filtration analysis

To analyze the mode of complex formation, a mixture of each type of hGH (intact 22 kDa hGH,

FIGURE 1. Gel filtration profile of the mixture of hGH and hGHBP. Concentrations of both hGHBP and hGHs (A, intact 22 kDa hGH; B, intact 20 kDa hGH; C, K157A; D, G105R) were all 0.5 μM . Mixtures were incubated for 15 min at 25 $^{\circ}\text{C}$ in 20 mM potassium phosphate buffer, pH 6.8, containing 0.05% Tween 20. Aliquots (50 μl) of the mixtures were applied to TSK-G2000SWXL column ($\Phi 7.8 \times 300$ mm) and eluted with the same buffer at 1.0 ml/min. Peaks were monitored on a fluorometer (Ex: 280 nm, Em: 340 nm). The ratio of each hGH isoform to recombinant hGHBP was fixed at 1:1 in the mixture. WT=wild type.

intact 20 kDa hGH, K157A or G105R) and hGHBP was subjected to gel-filtration. As shown in Fig. 1A, three peaks were observed in the mixture of 22 kDa hGH and hGHBP (molar ratio 1:1). These apparent sizes based on retention times corresponded to the 1:2 complex, 1:1 complex and free 22 kDa hGH. In contrast, the mixture of intact 20 kDa hGH and hGHBP (molar ratio 1:1) showed only two peaks, corresponding to the 1:2 complex and free 20 kDa hGH (Fig. 1B). It was also observed that K157A, the site1 mutant, formed a slight amount of the 1:2 complex and formed substantially no 1:1 complex (Fig. 1C), whereas G105R, the site2 mutant, only formed the 1:1 complex (Fig. 1D). These results indicated that 20 kDa hGH first bound hGHBP at site1 and that the resultant 1:1 complex immediately formed the 1:2 complex.

Ba/F3-hGHR cell proliferation

We have previously constructed a cell line of Ba/F3 stably expressing hGHR (Ba/F3-hGHR) and confirmed that 20 kDa hGH is a full agonist for the cell proliferation (Wada *et al.* 1998). To confirm altered hGHR-mediated actions of K157A and G105R, we measured cell-proliferation activities of

FIGURE 2. Ba/F3-hGHR cell proliferation caused by hGHs. Cells were dispensed in 96-well plates (4×10^4 cells/well) and incubated for 18 h in the presence of hGHs. The cell proliferation was measured by MT^T (triazolyl blue) assay. ●, 22 kDa hGH; ○, 20 kDa hGH; □, K157A; △, G105R. Each point represents the mean of triplicate wells, and error bars indicate s.d.

both mutants using the cell line. As compared with the intact form, the activities based on EC₅₀ values of K157A and G105R were reduced to 1/10 and 1/1000 respectively (Fig. 2). It was also observed that the activity of 20 kDa hGH was higher than that of 22 kDa hGH in the range of high ligand concentration (100 nM or more), although 20 kDa hGH showed a bell-shaped profile similar to 22 kDa hGH (Fig. 2).

Analysis of STEP1 affinity on biosensor

In 22 kDa hGH, residues 32–46 form C-terminal portion of helix1 and minihelix1 and are involved in binding to hGHR at site1. In 20 kDa hGH, this

region is deleted by alternative splicing. To examine effect of the deletion on the affinity for hGHR, we analyzed binding of both hGH isoforms to immobilized (S201C) hGHBP using biosensor. Since a molecule of the immobilized hGHBP could bind only one molecule of hGH or its mutant, the measured value can be considered as STEP1 affinity. As shown in Table 1, the STEP1 affinities (K_{d1}) of 22 kDa hGH, 20 kDa hGH and G105R were 2 nM, 16 nM, and 11 nM respectively. The K_{d1} value of 22 kDa hGH obtained here is compatible with that reported previously by Cunningham and Wells (1993). The binding of K157A to immobilized hGHBP could not be detected due to loss of the site1 affinity. The association rate constant (k_a) of STEP1 of 20 kDa hGH was almost the same as that of 22 kDa hGH (Table 1). The difference in STEP1 affinity between the hGH isoforms could be attributed to that of the dissociation rate constants.

Estimation of STEP2 affinity

Since it was impossible to measure STEP2 affinity directly on the biosensor we obtained the STEP2 affinity theoretically. The calculated relation between and receptor dimerization is shown in Fig. 3. Formation of the 1:2 complex was simulated by introducing experimentally obtained STEP1 affinities (K_{d1}) into equation (1), in which values of STEP2 affinities (K_{d2}) were calculatively altered. The simulated curves coincided with the experimentally obtained cell-proliferation curves reflecting receptor dimerization on the cell surface (Fig. 2) when the values 2.0 nM and 0.25 nM were adopted as K_{d2} values of 22 kDa hGH and 20 kDa hGH respectively (Fig. 3C).

Measurement of heat change in 1:2 complex formation

When the total binding affinities for receptor dimerization are the same, the change in Gibbs free

TABLE 1. Kinetic constants for binding of 22 kDa hGH or 20 kDa hGH isoforms to (S201C) hGHBP immobilized on the thiol matrix of the BIA core biosensor

Hormone	k_a ($s^{-1} M^{-1}$)	k_d (s^{-1})	K_{D1} (nM)
22 kDa hGH (WT)	2.7×10^5	5.1×10^{-4}	2
20 kDa hGH (WT)	1.7×10^5	2.8×10^{-3}	16
20 kDa hGH (K157A)	—	—	No binding
20 kDa hGH (G105R)	4.8×10^5	5.3×10^{-3}	11

The K_{D1} values were calculated as k_d/k_a . WT=wild type.

FIGURE 3. Calculated receptor dimerization curves corresponding to varied STEP2 affinities. Dose-response 1:2 complex formation curves were calculatively obtained according to equation (1) in the text. For each STEP1 affinity, the experimentally obtained value (Table 1) was used. (A) 22 kDa hGH; (B) 20 kDa hGH. $[hGH:(hGHP)_2]/[hGHP_0]$, molar ratio of formed 1:2 complex to initially presented free hGHP. (C) Calculated receptor dimerization curve compatible with the result of cell proliferation analysis shown in Fig. 2. Simulative curves of 22 kDa hGH (A) and 20 kDa hGH (B) coincide with the corresponding proliferation curves when STEP2 affinities (K_{d2}) are adopted as 2.0 nM (22 kDa hGH; ●) and 0.25 nM (20 kDa hGH; ○). For each STEP1 affinity, the experimentally obtained K_{d1} value shown in Table 1 was used.

energy during receptor dimerization should be equivalent in both hGH isoforms. To examine this assumption, we measured the heat change during addition of each hGH isoform into hGHP using a titration calorimeter. The signal of the heat change caused by hGH–hGHP binding could be observed up to the molar ratio 1:2 (hGH:hGHP) in each isoform (data not shown). As shown in Fig. 4, the obtained value of enthalpy change in 20 kDa hGH was statistically equivalent to that in 22 kDa hGH ($P>0.5$).

DISCUSSION

In this study we have first revealed the binding mode and affinities for hGHR of 20 kDa hGH and also clarified the K_{d2} value of 22 kDa hGH which

Hormone	ΔH (kcal/mol)	Molar ratio (hGH/hGHP)
22K-hGH(WT)	-24.4 ± 2.7	0.50 ± 0.04
20K-hGH(WT)	-25.0 ± 1.1	0.53 ± 0.01

FIGURE 4. Titration calorimetry data of the hGHP binding to 22 kDa hGH or 20 kDa hGH. Aliquot of hGHP solution was titrated with each hGH isoform. Protein solutions for calorimeter cells and injection syringes were dialyzed into 20 mM potassium phosphate buffer, pH 6.8, and titrations were carried out at 25 °C ($n=3$). Enthalpy was calculated using Microcal Origin program. WT=wild type.

had not been previously reported. As shown in Fig. 1, 20 kDa hGH substantially formed no 1:1 complex whereas its site2 mutant (G105R) only formed the 1:1 complex. This result suggests that 20 kDa hGH first binds hGHR at site1 and that the resultant complex immediately binds the second hGHR at site2. This assumption is compatible with the result that the site1 mutant (K157A) hardly formed the 1:1 complex. A small amount of the 1:2 complex in K157A (Fig. 1) suggests that the site1 affinity of this mutant partially remained. Slightly formed 1:1 complex in K157A shifts to 1:2 complex immediately. These results are consistent with the assumption that receptor dimerization of 20 kDa hGH proceeds in a sequential manner similar to that of 22 kDa hGH.

The results of reduced receptor dimerization of K157A and G105R are compatible with those of cell proliferation analysis in Ba/F3-hGHR (Fig. 2). In this cell proliferation analysis, 20 kDa hGH showed the bell-shaped activity curve similar to 22 kDa hGH, although it possessed higher activity than 22 kDa hGH in the range of high ligand concentration. This also strongly supports the assumption of the sequential binding and suggests that the diminished affinity for the first binding could permit reduced formation of the 1:1 complex on the cell surface under the condition of excess hGHR.

Binding analysis on the biosensor revealed that site1 affinity of 20 kDa hGH was reduced one-tenth as compared with that of 22 kDa hGH (Table 1). Based on the sequential binding model we have developed simulated curves of receptor dimerization corresponding to varied STEP2 affinities (Fig. 3) since the STEP2 affinity could not be experimentally determined. Superposition of the simulative curve on the cell proliferation curve indicated that both curves coincided with each other when STEP2 affinity (K_{d2}) of 20 kDa hGH and 22 kDa hGH were 0.25 nM and 2.0 nM respectively, indicating that the STEP2 affinity of 20 kDa hGH was approximately tenfold higher than that of 22 kDa hGH. This suggests that total binding affinity for receptor dimerization, which can be expressed as $K_{d1} \cdot K_{d2}$, could be almost the same in both isoforms. The result of calorimetric analysis revealed that enthalpy change associated with receptor dimerization of 20 kDa hGH was statistically the same as that of 22 kDa hGH. As shown in equation (3), the affinity constant for receptor dimerization can be determined by changed values of both enthalpy and entropy. Since the difference in the entropy change between both hGH isoforms can be negligible because of the structural similarity of whole molecules (Wada *et al.* 1998), the difference in affinity constants is mainly dependent on enthalpy changes. Based on this we concluded that the affinity constant of receptor dimerization in 20 kDa hGH was substantially the same as in 22 kDa hGH, which was consistent with the values of K_{d1} and K_{d2} of both isoforms determined above.

The results revealed that 20 kDa hGH possesses diminished STEP1 affinity ($K_{d1}=16$ nM) but shows increased STEP2 affinity ($K_{d2}=0.25$ nM) in comparison with 22 kDa hGH, possibly due to the conformational change of the 1:1 complex. When the concentration of hGH is sufficiently low compared with that of hGHR, receptor dimerization of hGH based on the sequential binding model can be expressed by equation (4). Under this condition, the concentration of the active 1:2 complex is in inverse proportion to overall affinity K_d that is derived from the product of K_{d1} and K_{d2} . Since the calculated K_d values of both hGH isoforms were the same (4.0×10^{-18} M²), 20 kDa hGH shows the same cell proliferation profile as 22 kDa hGH does under low concentrations. In contrast, under the condition of excessive hGH, receptor dimerization of hGH can be expressed by equation (5). This equation indicates that the more hGH concentration increases, the less the active 1:2 complex forms. This also implies that 20 kDa hGH forms active 1:2 complex more than 22 kDa hGH

does when the concentration of the hormone becomes sufficiently high in comparison with that of the receptor since the reduction of the 1:2 complex depends on K_{d1}/K_{d2} . The fact that we could not observe the formation of 2:2 (hGH:hGHBP) complex by gel-filtration analysis is consistent with the assumption and indicates that there is no possibility that the 1:1 complex binds with the other 1:1 complex.

$$[\text{hGH}:(\text{hGHBP})_2] = \frac{[\text{hGH}_0] \cdot [\text{hGHBP}_0]^2}{K_{d1} \cdot K_{d2}} \quad (4)$$

$$[\text{hGH}:(\text{hGHBP})_2] = \frac{[\text{hGHBP}_0]^2}{[\text{hGH}_0]} \cdot \frac{K_{d1}}{K_{d2}} \quad (5)$$

The diminished K_{d1} value of 20 kDa hGH is consistent with our previous result that 20 kDa hGH does not form the 1:1 complex in serum (Wada *et al.* 1998). Taken together, 20 kDa hGH exists as a free form in circulation and shows higher activity in the target tissue under the condition of high hGH:hGHR ratio as compared with 22 kDa hGH. It is possible that 20 kDa hGH is the isoform which can play an important role in the tissue expressing small amounts of hGHR or large amounts of short-form hGHR such as adipose tissues.

Since the site2 region of 20 kDa hGH is considered to be conformationally the same as that of 22 kDa hGH (Wada *et al.* 1998), the increased STEP2 affinity might be attributed to the reinforced binding affinity between hGHBP at the stem portion. In 22 kDa hGH there are several reports suggesting that the transition state between the 1:1 complex and the 1:2 complex requires not only the affinity of the site2 region but also association of stem regions of two hGHRs. Crystallographic studies on the 1:2 complex have revealed that there is a substantial contact surface between the C-terminal domains of hGHBP (De Vos *et al.* 1992, Wells 1996) and that residues 145S, 146L and 147T located in the stem region of hGHR (C-terminal domain of hGHBP) are stabilized by dimerizing (Clackson *et al.* 1998). The results of mutational experiments have also indicated that the stem region of hGHR is involved in effective signal transducing (Chen *et al.* 1997).

It is possible that binding of 20 kDa hGH to the first hGHBP causes conformational change of the 1:1 complex that leads to close contact of stem regions of hGHBP and consequently provides increased affinity (Fig. 5). Here we tentatively designate the stem region of hGHR involved in receptor dimerization 'siteBP'. As for 22 kDa hGH, Clackson *et al.* (1998) have reported that conformational changes in ligand or receptor do not play a

FIGURE 5. Schematic illustration of the mode of receptor dimerization of both hGH isoforms.

role in the transition from the 1:1 to the 1:2 complex. Further investigation is necessary to clarify whether the difference of STEP2 affinity (K_{d2}) between 20 kDa hGH and 22 kDa hGH is dependent on conformational change of siteBP in the 1:1 complex. Crystallographic analysis and mutational analysis of the siteBP region are now in progress.

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