

Dimerization of the Extracellular Domain of the Human Growth Hormone Receptor by a Single Hormone Molecule

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Human growth hormone (hGH) forms a 1:2 complex with the extracellular domain of its receptor-binding protein (hGHbp) as studied by crystallization, size exclusion chromatography, calorimetry, and a previously undescribed fluorescence quenching assay. These and other experiments with protein engineered variants of hGH have led to the identification of the binding determinants for two distinct but adjacent sites on hGH for the hGHbp, and the data indicated that there are two overlapping binding sites on the hGHbp for hGH. Furthermore, the binding of hGH to the hGHbp occurred sequentially; a first hGHbp molecule bound to site 1 on hGH and then a second hGHbp bound to site 2. Hormone-induced receptor dimerization is proposed to be relevant to the signal transduction mechanism for the hGH receptor and other related cytokine receptors.

HORMONE-INDUCED RECEPTOR OLIGOMERIZATION HAS been proposed as a mechanism of signal transduction for a large family of tyrosine kinase receptors that contain an extracellular hormone binding domain (for example, for platelet-derived growth factor or epidermal growth factor, EGF), a single transmembrane segment and a cytoplasmic tyrosine kinase domain (1). In models for these receptors, binding of one hormone molecule (or subunit) (H) per receptor (R) is believed to induce formation of an H_2R_2 complex. Binding is followed by activation of the intracellular tyrosine kinase and receptor autophosphorylation. More recently, another family of single transmembrane hormone receptors has been discovered; this family includes the human growth hormone (hGH), human prolactin (hPRL), and at least eight other cytokine and hematopoietic receptors (2). These receptors contain homologous extracellular hormone binding domains and highly variable intracellular domains that are not homologous to any known tyrosine kinase or other protein.

To analyze the structural requirements and mechanism (or mechanisms) for hormone-induced changes in these hormone receptors, we have prepared in high yield the extracellular domain of the hGH receptor (called hGHbp) from an *Escherichia coli* gene expression

system (3). The specificity and affinity for hGH of the highly purified and nonglycosylated hGHbp is essentially the same as that of the natural hGHbp found in serum ($K_D \sim 0.4$ nM). We have now identified the binding determinants and mechanism for formation of a signal transduction complex containing only one molecule of hGH and two molecules of the hGHbp.

Formation of the $hGH \cdot (hGHbp)_2$ complex in crystals and in solution. Crystals of the complex between hGH (22 kD) and the hGHbp (28 kD) were grown by vapor phase diffusion (4). The composition of the crystals was analyzed by dissociation in 0.1 percent trifluoroacetic acid and chromatography under denaturing conditions (Fig. 1). From four separate chromatograms, the ratio of the integrated absorbance at 214 nm of the hGH peak to the hGHbp peak was 0.42 ± 0.02 . For a complex having the form $hGH \cdot (hGHbp)_2$, the ratio predicted for integrated peak areas is 0.40, based on the number of peptide bonds in each of the components (191 residues for hGH and 238 residues for the hGHbp) (5).

Next, we used isothermal titration calorimetry to evaluate the

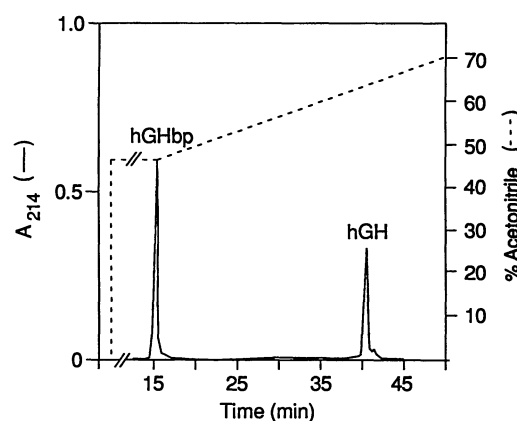


Fig. 1. High-performance liquid chromatography (HPLC) profile of crystalline complex dissociated and analyzed on a Vydac C_{18} (5 μ m, 4.6 by 150 mm) column equilibrated in 10 percent acetonitrile containing 0.1 percent trifluoroacetic acid. The components were eluted isocratically with a linear acetonitrile gradient at a flow rate of 1 ml/min. The gradient was started at the arrow and is illustrated with a dashed line; the peptide bond absorbance at 214 nm is represented by the solid line. The complex was prepared by adding a slight excess of hGH to hGHbp, and purifying the complex over a sephadex G75-120 size exclusion column equilibrated in 10 mM tris (pH 8.0) and 100 mM NaCl. The high molecular size peak, containing the complex, was separated from free hGH, pooled and concentrated. Crystals of complex were grown from 20 percent saturated ammonium sulfate solution at pH 7.5 by vapor diffusion technique (4).

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stoichiometry of the complex in solution. Portions of hGH were added to a solution containing a fixed concentration of the hGHbp (15 μ M) (6) and the heat of reaction was measured until there was no further enthalpic change (Fig. 2). The average ratio of hGH to hGHbp necessary for binding was 0.46 ± 0.05 ($n = 3$) and the ΔH was about -27 kcal per mole of hGH.

The size and stoichiometry of the complex was further established by separating mixtures of hGHbp and hGH (in ratios of 4:1, 3:1, 2:1, 1:1, and 0.5:1) by gel filtration (Fig. 3A). At a 2:1 ratio of hGHbp to hGH, virtually all the protein chromatographed as a 75-kD complex. SDS-PAGE and densitometry of protein samples taken across the complex peak confirmed that the complex contained 2 mol of hGHbp per mol of hGH (7). When the ratio of hGHbp to hGH was greater than 2:1, excess free hGHbp was present; at ratios less than 2:1, free hGH was present as well as the monomeric complex hGH $\cdot$ hGHbp. The areas of the peaks were dominated by the absorbance of the hGHbp because its $\epsilon_{280}^{0.1\%}$ (extinction coefficient) was 2.9 times higher than hGH (6). Additional control experiments showed that the free components behaved as monomeric proteins, an indication that high affinity dimerization of the hGHbp requires the presence of hGH.

Evidence for two receptor binding sites. The binding of human prolactin (hPRL) or human placental lactogen (hPL) to the hGHbp is more than 10^5 or 10^3 times weaker than that of hGH, respectively. However, by incorporating eight substitutions into hPRL (E62S, D63N, Q66E, H171D, E174A, N175T, Y176F, and K178R) (8) or five into hPL (V4I, D56E, M64R, E174A, and M179I) (9) we produced variants in which the binding to the hGHbp is only six or four times weaker than to hGH, respectively. We used gel filtration to determine whether these variants could bind to one or two molecules of the hGHbp (Fig. 3B). Two symmetrical peaks appear at a 2:1 ratio of hGHbp to either the hPRL (24 kD) or hPL (22 kD) variants. One corresponds to a 1:1 complex with the hGHbp (approximately 55 kD) and the other peak represents a stoichiometric excess of the hGHbp (20 kD). Peak compositions were confirmed by SDS-PAGE (7). Furthermore, the titration calorimetry end point of the hPRL variant with the hGHbp was reached at a ratio of 0.85:1, respectively (10).

These data indicate that the hPRL and hPL variants were missing important determinants for dimerization of the hGHbp and suggest there were two binding sites on hGH for the hGHbp. One of these sites (called site 1) has been functionally characterized in detail by

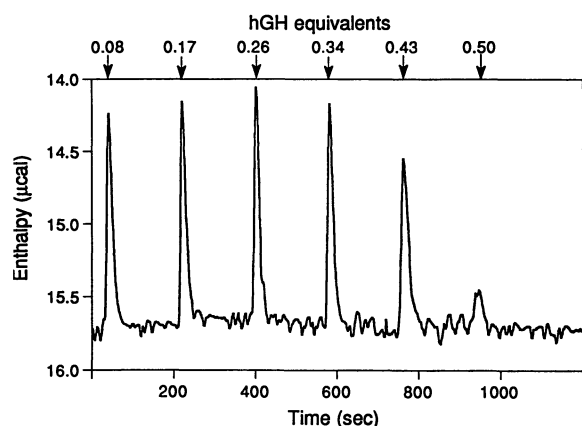


Fig. 2. Titration calorimetry of hGH with hGHbp. The hGHbp [at 15 μ M in 10 mM tris (pH 8.0)] was placed in a 1.37-ml titration cell (MC2 titration calorimeter, Microcal, Northampton, Massachusetts) and equilibrated at 25°C. A solution containing hGH [437 μ M in 10 mM tris (pH 8.0)] was added in 4- μ l injections over 8 seconds with an interval of 5 minutes between each injection. The heat effects for injection of buffer lacking hGH has been subtracted from the profile.

alanine-scanning mutagenesis of hGH (11). The second sites on hGH were not identified because the monoclonal antibody to the receptor, MAb5, which was used to precipitate the complex, only allowed formation of a monomeric complex.

The affinity of hGH for the hGHbp is typically measured by displacement of 125 I-labeled hGH from the hGHbp, and precipitation of the complex with MAb5 produced from rabbit glycosylated GH receptor (12). Analyses of these binding isotherms (Fig. 4) show that hGH and the hGHbp form a 1:1 complex when precipitated with MAb5. Additional monoclonal antibodies have been produced by immunization with the unglycosylated hGHbp purified from *E. coli* (3, 13). Binding analysis with two of these monoclonal antibodies to hGHbp (MAb 3B7 and MAb 3D9) give dissociation constants of about 0.2 nM and stoichiometries of about 0.5 hGH per hGHbp (Fig. 4). These results indicate that MAb 3B7 and 3D9 do not block determinants on the hGHbp for dimerization whereas MAb 5 does.

The lack of cooperativity in assays with MAb 3B7 and MAb 3D9 may reflect the independence of the binding sites on hGH, or that the affinity of hGH in the 1:1 complex (as measured with MAb 5) is nearly that for the 2:1 complex (as measured with mAb 3B7 or 3D9). Moreover, MAb 3B7 and 3D9 should precipitate the same amount of 125 I-labeled hGH in either the 1:1 or 2:1 complexes, which would dampen any apparent cooperativity.

Binding determinants for the second receptor site on hGH. A sensitive solution assay based upon fluorescence "homoquenching" was developed to study the hormone-induced dimerization of the hGHbp without the need for immunoprecipitation. In this assay fluorescein undergoes homoquenching when two molecules come close together (14). An hGHbp mutant (S237C) with a free thiol at its penultimate residue was covalently coupled to 5-iodoacetamido-

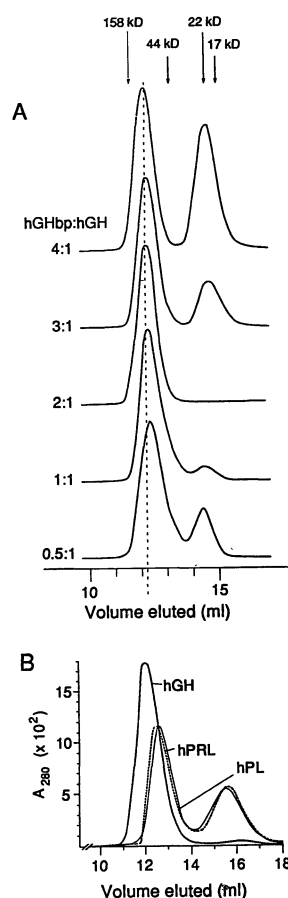
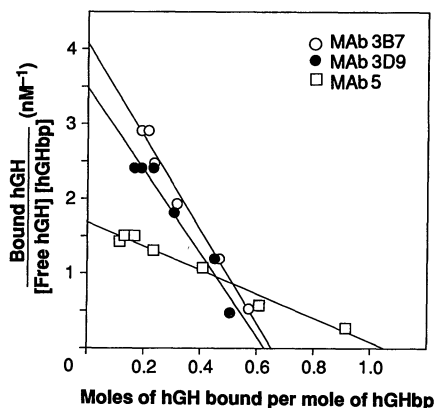


Fig. 3. (A) Gel filtration chromatography of a various ratios of hGHbp to hGH corresponding to 4:1, 3:1, 2:1, 1:1, and 0.5:1 (top to bottom tracings). The concentrations of hGH (fixed at 10 μ M except at 1:1 and 0.5:1 ratios, where hGH was 20 μ M and 40 μ M, respectively) and hGHbp in each mixture were determined by absorbance at 280 nm (6). Protein mixtures were equilibrated for 15 minutes at 25°C in 20 mM tris (pH 7.5), 100 mM NaCl. Samples (100 μ l) were applied to a Superose 12 FPLC column (Pharmacia) and eluted with the same buffer at 0.35 ml/min. Peaks were monitored for absorbance at 280 nm. (B) Gel filtration chromatography of a 2:1 ratio of the hGHbp to hGH (—) and variants of hPRL (-----) or hPL (---) that were engineered to bind to the hGHbp (8, 9).

Fig. 4. Analyses for the binding of hGH to the hGHbp in the presence of various monoclonal antibodies to hGHbp. A fixed amount of ^{125}I -labeled hGH and increasing amounts of unlabeled hGH were allowed to equilibrate overnight in the presence of hGHbp (fixed at 165 pM) and 30 nM of each mAb. The hGHbp and complexes with hGH were precipitated with polyethylene glycol, and the precipitates were counted as described (3, 11). Reformulation of the displacement data in Scatchard form shown above permits a calculation of the apparent dissociation constants for binding of hGH to the hGHbp in the presence of MAb 5, 3B7, and 3D9 of 0.62 nM, 0.17 nM, and 0.19 nM, and saturation stoichiometries of hGH to hGHbp of 1.01, 0.64, 0.64, respectively.



fluorescein (5-IAF) (15). This highly fluorescent adduct (called S237C-AF) contained one fluorescein molecule covalently linked near the site of attachment of the hGHbp to the transmembrane segment in the case of the full-length hGH receptor. Binding analysis (7) showed that the S237C-AF has the same affinity for site 1 on hGH as unmodified hGHbp when tested with the MAb 5 immunoprecipitation assay (Fig. 4).

When hGH was added to the S237C-AF, we observed fluorescence quenching that was maximal when 0.5 molar equivalents of hGH was added (Fig. 5). We have demonstrated Förster energy transfer with a nonidentical donor-acceptor-pair in which the fluorescence quenching of a donor (S237C-AEDANS) was coupled to the fluorescence (S237-AF) enhancement of an acceptor on addition of hGH (16). We prefer the homoquenching assay with S237C-AF because of its greater sensitivity and its simplicity in the interpretation of a homodimeric complex.

In six separate experiments, we obtained a median effective concentration (EC_{50}) of $0.54 (\pm 0.14)$ nM for serial dilutions of a 1:2 mixture of hGH to S237C-AF (Fig. 5). This value is close to the dissociation constant determined by immunoprecipitation with MAb 3B7 (Fig. 4) for formation of a 2:1 complex (0.2 nM). Furthermore, the hPL and hPRL analogs that bind but do not dimerize the hGHbp (Fig. 3) do not induce homoquenching of S237C-AF (7). Thus, the fluorescence quenching dimerization assay is highly sensitive, specific, and reflects accurately the formation of the $\text{hGH} \cdot (\text{hGHbp})_2$ complex in solution.

We used the fluorescence dimerization assay to identify binding determinants on hGH for the second molecule of hGHbp. As expected, we found that mutants of hGH in site 1 disrupted dimerization of the hGHbp as monitored in this assay (7). We then tested a series of hGH mutants with deletions, hPL, or hPRL segment substitutions (17) in regions outside of site 1 (Table 1). Two of these variants [$\Delta 1-8$ and hPRL(111-129)] caused large reductions (>100 times lower) in the EC_{50} for dimerization with little or no effect on binding to site 1.

Alanine-scanning mutagenesis (11) was used to identify side chains near the amino-terminus (residues 1-8) or displayed from the hydrophilic face of helices 1 and 3 (residues 8-21 and 109-128) important for hGH-induced dimerization of the hGHbp (Table 2). We also probed side chains at positions 186 and 188 because we did not have an appropriate segment substituted analog in the extreme carboxyl-terminal region. Three of the most disruptive substitutions (F1A, I4A, and R8A) were located in the NH_2 -terminal segment.

The α -amine of Phe¹ does not appear to be important because Met-hGH, which contains an additional NH_2 -terminal Met residue, has the same EC_{50} as wild-type hGH (7). Furthermore, an hGH derivative that deletes Phe¹ and Pro² has the same EC_{50} value as variant F1A, an indication that the phenylalanyl side chain of Phe¹ is the moiety important for binding (18). Despite the large disruption observed for the hPRL(111-129) variant in helix 3, only D116A produced a substantial reduction in binding, which was followed by a mild effect for E119A. Either the importance of helix 3 determinants is exaggerated because of conformational effects in the hPRL(111-129) and hPL(109-112) mutants, or many side chains contribute weakly to binding and, when substituted individually to Ala, give virtually wild-type binding.

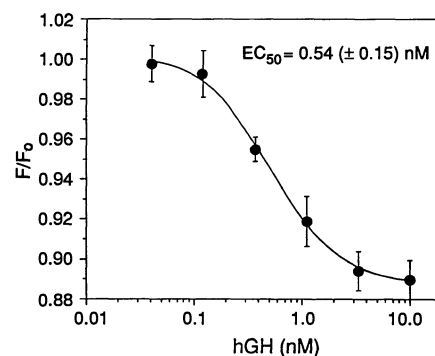
When the disruptive alanine substitutions were mapped on a model of hGH, which is based on homology to the porcine GH structure (19), they formed a patch adjacent to that for site 1 (Fig. 6). It is possible that the patch size for site 2 appears smaller than that for site 1 because some site 2 determinants may have been missed in our screen. However, it is more likely some of the site 2 determinants arise from interactions between the two bound molecules of hGHbp (20).

Sequential binding of hGHbp to site 1 on hGH then site 2.

The addition of hGH to the $\text{hGH} \cdot (\text{hGHbp})_2$ complex generated a 1:1 complex ($\text{hGH} \cdot \text{hGHbp}$) by gel filtration (Fig. 3A). We then used the fluorescence assay to investigate the hGH-induced dissociation of the $\text{hGH} \cdot (\text{hGHbp})_2$ complex. Titration of S237C-AF with hGH beyond that required for formation of the $\text{hGH} \cdot (\text{S237C-AF})_2$ complex (0.5 molar equivalent) reversed the observed fluorescence quenching (Fig. 7) with an EC_{50} of about 30 nM at 6 times greater molar excess (Fig. 7). This shows that excess hGH can dissociate the $\text{hGH} \cdot (\text{S237C-AF})_2$ complex and is consistent with the hGHbp containing two overlapping sites for binding hGH.

To identify which binding site is used to form the $\text{hGH} \cdot \text{hGHbp}$ complex under conditions of excess hGH, we examined two hormone variants that have just one functional site (Fig. 7). The hPL variant (9), which contains a functional site 1 but lacks a functional site 2, dissociated the $\text{hGH} \cdot (\text{hGHbp})_2$ complex as efficiently as hGH ($EC_{50} = 20$ nM; a fourfold hormone excess over hGHbp). By contrast, an hGH double mutant (K172A, F176A) that retains site 2 determinants but lacks the ability to bind tightly to the hGHbp at

Fig. 5. Determination of EC_{50} for hGH-induced dimerization of S237C-AF by fluorescence homoquenching. Serial dilutions (threefold) of S237C-AF (15) in binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 percent BSA, 0.02 percent NaN_3) were made over a range from 20 nM to 0.08 nM and 1.0-ml portions were dispensed to polypropylene mixing tubes (12 by 75 mm). Similarly, hGH was serially diluted, but over a range from 1 to 0.004 μM . Portions (10 μl) of each hGH dilution to finally give a 1:2 molar ratio of hGH to S237C-AF (or buffer only) were added to the S237C-AF in mixing tubes. The mixtures were incubated for 6 hours at 25°C in the dark. Fluorescence measurements were made in cuvettes at an excitation wavelength (λ) of 490 nm and an emission wavelength (λ) of 512 nm (bandwidths were 3 nm and 10 nm, respectively) with the use of a Shimadzu RF5000U spectrofluorophotometer. Each data point represents the mean of six measurements ($\pm$ SEM) from independent experiments. An average EC_{50} of $0.54 (\pm 0.14)$ nM was calculated as the concentration of hGH giving half-maximal $\Delta F/F_0$ values as determined from a 4-parameter curve fit.



site 1 (500 times lower affinity) (21) was unable to reverse dimerization even when present at 800 nM (160-fold hormone excess). These data show that the dissociation of hGH · (hGHbp)₂ complex by excess hGH was mediated through site 1, not site 2.

Our data suggest that hGH-induced dimerization of the hGH receptor occurs through a sequential binding mechanism. In step 1, an hGH · hGHbp complex is formed at site 1, and then an additional hGHbp is bound at site 2 to give hGH · (hGHbp)₂. The existence of the site 1 intermediate (hGH · hGHbp) is shown by dissociation of the hGH · (S237C-AF)₂ complex by functional site 1 variants (Fig. 7). Furthermore, the formation of the hGH · hGHbp complex via site 1 can be trapped by MAb 5 which blocks dimerization (Fig. 4), or hPL and hPRL variants which only have a functional site 1 (Fig. 3B). We see no evidence for formation of an hGH · hGHbp complex mediated exclusively through site 2. Together, our data indicate that there are two sites on hGH that sequentially bind two corresponding and overlapping sites on the hGHbp.

Evidence that dimerization is important for activation of the hGH and other cytokine receptors. The hGH and hPRL receptors are structurally homologous, and hGH can bind and activate both receptors. Recently, it has been shown that monoclonal antibodies (and not a corresponding Fab fragment) to the rat PRL receptor induce proliferation of rat lymphoma Nb2 cells (22). We have obtained data showing that a monoclonal antibody to the rat receptor is capable of promoting weight gain (23). These data suggest that receptor oligomerization is associated with the biological activities of hGH in cells and in whole animals. Also, an analog missing the first seven residues of hGH was shown to have reduced affinity for receptors on IM-9 cells and decreased receptor down regulation at saturation (24). Our results [Δ(1–8), Table 1] suggest that this is caused by loss in binding at site 2. Together, these data indicate that nondimerizing analogs of hGH should antagonize the biological effects of hGH *in vivo*.

The extracellular domains of the hGH and hPRL receptors are homologous to a large family of cytokine receptors including IL-2, IL-3, IL-4, IL-6, IL-7, erythropoietin, G-CSF, and GM-CSF receptors (2). Although the signal transduction mechanism for these

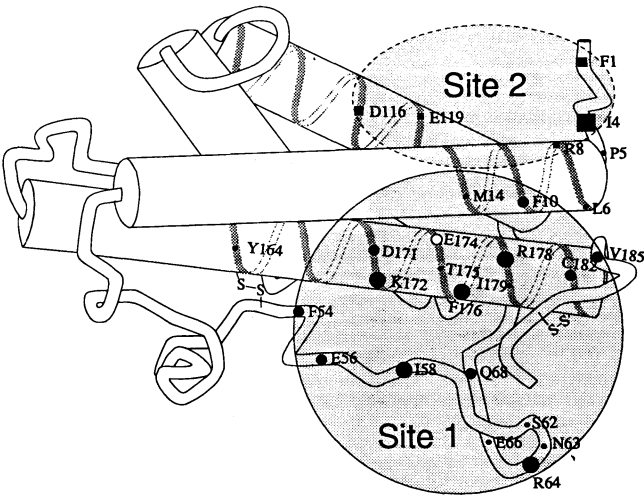


Fig. 6. Map of alanine substitutions in hGH that disrupt binding of hGHbp at either site 1 or site 2. The two sites are generally delineated by the large shaded circles. Residues for which alanine mutants reduce site 2 binding by two- to fourfold, four- to tenfold, ten- to fiftyfold, and more than fiftyfold are shown by graduated squares (■, ▤, ▥, and ▦), respectively. Residues marked by the symbols, ●, ●, ●, and ○, represent sites where Ala mutations in site 1 of hGH cause reductions of two- to fourfold, four- to tenfold, greater than tenfold, or a fourfold increase in binding affinity for the hGHbp, respectively using the MAb 5 immunoprecipitation assay (8, 11). The model of hGH is based on structure of pGH (19).

Table 1. EC₅₀ values for the dimerization of S237C-AF induced by various segment substitutions derived from homologs (hPL or hPRL) or deletion mutants (Δ) or hGH. The beginning and ending residues in the homolog substitutions or deletions are given in parentheses. The specific identities of the mutants and their respective K_D values for site 1 binding have been described (17). IC₅₀ values are determined as described in Fig. 5.

hGH mutant	hGHbp dimerization		Site 1
	EC ₅₀	EC ₅₀ (mut)	hGHbp
		EC ₅₀ (wt)	binding
			K _D (mut)
			K _D (wt)
wt hGH	0.54	(1)	(1)
Δ(1–8)	≥50	≥100	4.4
hPRL(22–33)	0.66	1.2	0.9
Δ(32–46)	0.42	0.8	ND
hPRL(88–95)	0.72	1.3	1.4
hPRL(97–104)	1.6	2.9	1.6
hPL(109–112)	3.0	5.5	1.8
hPRL(111–129)	≥50	≥100	1.5
hPRL(126–136)	1.2	2.2	1.7
hPRL(137–145)	0.69	1.3	ND
hPRL(146–152)	0.51	0.9	ND

class 1 cytokine receptors is unknown, there is evidence that after initial binding of IL-2 or IL-3 to their receptors (α subunit), an accessory receptor (β subunit) may bind to mediate the biological signal (25, 26). This binding sequence appears analogous to the sequential mechanism for receptor dimerization that we propose for hGH signal transduction. In addition, many of these cytokines share a four-helical-bundle structural motif with GH proteins (2) and their binding sites may be similar to those in hGH.

The extracellular domain of the EGF receptor (EGFbp) has been crystallized in a 1:1 complex with EGF (27). Binding studies and sedimentation analysis showed that EGF forms only a 1:1 complex with the EGFbp in solution, suggesting that the EGFbp is insufficient to undergo hormone-induced dimerization. However, the binding studies used polyclonal antibodies to the EGF receptor to

Table 2. EC₅₀ values for dimerization of S237C-AF induced by alanine substituted hGH mutants. Mutants were prepared as described (8, 11, 21). A mutant not expressed is designated NE. EC₅₀ numbers are calculated as described in Fig. 5. Standard deviations are generally less than ±35 percent. The integrity of all mutant proteins with EC₅₀ values at least three times greater than those of the wild-type was verified by NH₂-terminal sequencing and electrospray ionization mass spectrometry.

hGH mutant	Dimerization EC ₅₀	EC ₅₀ mutant	hGH mutant	Dimerization EC ₅₀	EC ₅₀ mutant
		EC ₅₀ wt			EC ₅₀ wt
wt hGH	0.54	—	H21A	0.51	1.0
F1A	2.9	5.4	D107A	0.38	0.7
P2A	0.58	1.1	N109A	0.35	0.7
T3A	0.72	1.3	Y111A	1.0	1.9
I4A	30	55	D112A	0.53	1.0
P5A	0.92	1.7	K115A	0.84	1.6
L6A	1.4	2.5	D116A	3.1	5.7
S7A	0.37	0.7	E118A	0.96	1.8
R8A	1.8	3.4	E119A	1.1	2.0
F10A	0.77	1.4	Q122A	0.4	0.7
D11A	NE	—	T123A	0.65	1.2
N12A	0.59	1.1	R127A	0.80	1.1
L15A	0.36	0.7	E129A	0.70	1.3
R16A	0.63	1.2	D130A	0.42	1.8
H18A	0.55	1.0	E186A	0.58	1.1
R19A	0.92	1.7	S188A	0.49	0.9

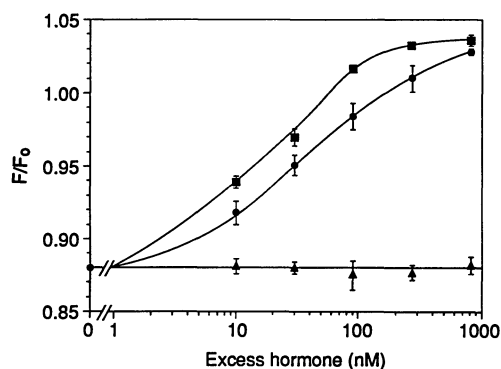


Fig. 7. Dissociation of the hGH · (S237C-AF)₂ complex by excess hGH or hGH mutant. S237C-AF and hGH were present at a concentration of 10 nM and 5 nM, respectively, and 1.0-ml samples were dispensed to assay tubes. Serial dilutions of either wild-type hGH, mutant, or buffer only were added, and the mixtures were incubated for 6 hours at 25°C in the dark. Fluorescence was measured as described for Fig. 5. Data points are means of triplicate measurements for binding of hGH, ●, K172A, F176A, ▲ (20); hPL variant, ■ (9) to S237C-AF. The increase in F/F_0 above 1.0 at large excess of hGH (>70 nM) reflects greater nonspecific binding of free S237C-AF (F_0) compared to bound (F) in the assay tubes, which occurs during the equilibration phase prior to the fluorescence measurements. Although this effect slightly distorts the EC_{50} values, the relative values, which are the basis of our comparisons, should remain unaffected.

precipitate the complex. Furthermore, the crystallization and sedimentation reaction mixtures contained a large excess of hormone over receptor. In our case, a monoclonal antibody to the natural GH receptor can block dimerization (Fig. 4) and a large excess of hGH dissociates the hGH · (hGHbp)₂ complex into a monomeric complex (Fig. 7). Recently, electron micrographs showing that EGF can induce oligomerization of the EGFbp have been described (28). From analysis (28) for binding of EGF to the EGFbp (where complexes were separated with a MAb to the EGF receptor), we calculated a stoichiometry of about 1:2 EGF:EGFbp—that is, 2.8 nM EGF bound to 6.5 nM EGFbp at saturation (Fig. 2) (28). This ratio could be in error if the ratio of native EGFbp to EGF were grossly different than the total concentrations reported.

We conclude that hGH contains two functionally distinct sites for binding to two overlapping sites on the hGHbp in producing the hGH · (hGHbp)₂ complex. We suggest that formation of the analogous dimeric receptor complex on the cell surface is critical to the signal transduction mechanism of hGH (and perhaps other homologous cytokine receptors). These assays should be applicable to the study of other receptors in which dimerization is believed to be important in signal transduction. These functional studies are complementary to high-resolution structural analysis, and together may allow us to begin designing small molecule agonists and antagonists of hGH.

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3. G. Fuh et al., *J. Biol. Chem.* **265**, 3111 (1990). The frozen cell paste was thawed in hypotonic buffer [10 mM tris (pH 8.0), 1 mM PMSF (Sigma), 2 mM EDTA]. The suspension was homogenized, stirred for 1 hour at 4°C, and then centrifuged at 10,000g for 20 minutes. Solid ammonium sulfate was added to 260 g/l, and the protein precipitate was collected by centrifugation at 10,000g for 30 mins. The pellet was resuspended in 10 mM tris (pH 8.0) and 1 mM PMSF, and dialyzed against the same buffer. The dialysate was applied to a Q Sepharose column (Pharmacia) in 10 mM tris (pH 8.0) and eluted with a linear gradient of 0.0 to 0.5

- M NaCl. Peak fractions containing the hGHbp were placed directly on an hGH affinity column. The column was washed and then eluted with 4 M MgCl₂, 10 mM tris (pH 7.5). The peak fractions were combined and dialyzed with 10 mM tris (pH 7.5) and applied to a Mono Q column. The column was washed and eluted in 10 mM tris (pH 7.5) with a linear gradient of 0 to 0.2 M NaCl.
4. A. McPherson, in *Preparation and Analysis of Protein Crystals* (Krieger, 1989) The crystals diffract to 2.7 Å and belong to space group (P2₁, 2₁, 2) with unit cell parameters of $a = 145.8$ Å, $b = 68.6$ Å, and $c = 76.0$ Å. The volume of the asymmetric unit of these crystals is such that the complex is unlikely to have the form of either hGH · hGHbp or (hGH · hGHbp)₂. In particular, the solvent content would be 68 percent for a 1:1 complex or 35 percent for a 2:2 complex in the asymmetric unit, compared to the typical solvent content of protein crystals of about 50 percent [B. W. Matthews, *J. Mol. Biol.* **33**, 491 (1968)].
5. In control experiments, a 1:2 mixture of hGH to the hGHbp produced essentially the same chromatogram as in Fig. 1, whereas 2:1 and 1:1 mixtures generated expected and different chromatograms.
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15. The hGHbp mutant (S237C) was constructed and purified as described [S. Bass, R. Greene, J. A. Wells, *Proteins* **8**, 309 (1990)] (3). A solution of S237C at 1 mg/ml was brought to 25 mM cysteine · HCl, 25 mM NaHCO₃ and incubated for 2 hours at 4°C to ensure reduction of Cys²³⁷. DTNB analysis of the reduced S237C prior to reaction with 5-IAP showed an average of one free thiol per S237C molecule (22 μ M free SH per 17 μ M S237C). A similarly treated wild-type hGHbp yielded less than 0.1 free thiol per hGHbp, an indication that these reduction conditions do not effectively reduce either of the three disulfide bonds. The protein was desalted with a PD10 (Pharmacia) minicolumn [equilibrated with 50 mM tris-HCl (pH 8)] and immediately reacted with 500 μ M 5-IAP (Molecular Probes) for 16 hours at 4°C in dark. The S237C-AF was purified from free fluorophore with the use of another PD10 minicolumn equilibrated with 20 mM tris-HCl (pH 7.5). Portions of purified S237C-AF were stored at -80°C and thawed just before use. Absorption spectral analysis of the S237C-AF shows 0.84 μ M fluorescein bound per 1.0 μ M S237C based on molar extinction coefficients of 71,300 (at 494 nm) and 64,800 (at 280 nm) and corrections for interfering 5-IAP adsorbance at 280 nm (14).
16. S237C-AEDANS was prepared essentially as described for S237C-AF (15) by reaction with 1,5 Br-AEDANS [5-[[2-(bromoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid. Förster energy transfer was monitored at an excitation wavelength (λ) of 360 nm; donor fluorescence was measured at 460 nm and acceptor fluorescence was measured at 512 nm.
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