Growth Hormone Binding Affinity for Its Receptor Surpasses the Requirements for Cellular Activity

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ABSTRACT: The human growth hormone (hGH)−receptor interaction was used to study the relationship between hormone−receptor affinity and bioactivity. hGH has two nonequivalent sites, called site 1 and site 2, that bind two molecules of receptor in a sequential fashion. We produced both site 1 and site 2 high-affinity hGH variants either by combining alanine mutants previously found to improve affinity at site 1 or by random mutagenesis of residues in site 2 followed by phage display and receptor binding selections. The two high-affinity variants, as well as one which combined them, were used in cell proliferation assays with FDC-P1 cells expressing the hGH receptor. Interestingly, none of these variants produced a change in the EC_{50} for cell proliferation or the levels of JAK2 tyrosine kinase phosphorylation. Next we studied the effect of a reduction in site 1 affinity on cell proliferation. A systematic series of hGH mutants were produced in which affinity for site 1 was reduced from 5- to 500-fold. Surprisingly, the EC_{50} for cell proliferation was unaffected until affinity was reduced about 30-fold from wild-type hGH. Thus, native hGH−receptor affinity is much higher than it needs to be for maximal JAK2 phosphorylation or cell proliferation. These studies begin to define basic functional tolerances for receptor activation that need to be considered in the design of hGH mimics.

Virtually all single membrane-spanning hormone receptors are believed to be activated by receptor oligomerization (*1*). The cytokine receptor superfamily, which includes the receptors for growth hormone and erythropoietin, is a paradigm for single-pass membrane receptors that form oligomeric complexes upon hormone binding (*2*). hGH[1] is a monomeric hormone and has two nonidentical sites, called site 1 and site 2. These bind two receptors in an ordered fashion first through site 1 and then through site 2 (*3*−*5*). Receptor dimerization initiates phosphorylation of the Janus tyrosine kinase, JAK2 (*6*). Activated JAK2 then phosphorylates STAT3 among other proteins and stimulates transcription of new gene products leading to cell proliferation and other effects (*7*).

In developing analogues or mimics of hGH, it is important to understand how hormone binding affinity relates to ability to stimulate a biological response. Such studies contribute fundamental information that links simple binding events at the cell surface with more complex signal transduction and transcriptional activation processes downstream. Thus, we were interested in producing higher affinity hGH mutants and characterizing their abilities to initiate signal transduction and cell proliferation. If receptor activation and cell proliferation are controlled by simple equilibrium binding, it is predicted that a higher affinity site 1 variant would yield a lower EC_{50} for cell proliferation and that a higher affinity site 2 variant would yield a higher maximum response than wild-type hGH (*8*, *9*).

Growth hormone analogues having higher receptor binding affinity at site 1 have been produced by alanine-scanning mutagenesis (*10*, *11*) or by monovalent phage display (*12*−*14*). Using these methods, variants of hGH were generated whose affinities were improved about 50-fold for site 1, site 2, or both. The three affinity-improved mutants were used in cell proliferation and JAK2 intracellular kinase activation assays to test the model predictions. None of the analogues, nor ones with reduced site 1 affinity, caused a significant improvement in potency in either bioassay. Thus, hGH binding affinity surpasses that required for maximal cellular activity.

MATERIALS AND METHODS

General Procedures. hGH mutants were constructed by site-directed mutagenesis (*15*), and expressed in *Escherichia coli* (*11*). For use in cell-based assays, proteins were purified to homogeneity by gel filtration (Sephacryl-100 HR; Pharmacia Biotech, Inc., Uppsala, Sweden) and hydrophobic interaction chromatography (Toyopearl phenyl 650M; Toso-Haas). Mass spectrometry was used to verify the mass of all purified variant proteins. Concentrations of purified proteins were determined by OD_{280} using ϵ_{280} = 15 782, 15 592, 26 652, and 26 736 (M^{-1} cm^{-1}) for hGH_{wt}, octa-

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[1] Abbreviations: hGH, human growth hormone; hGHbp, extracellular domain of the human growth hormone receptor; PBS, phosphate-buffered saline; RU, resonance unit; FBS, fetal bovine serum. Mutant proteins are named with the single-letter code for the wild-type residue, followed by the amino acid position and the single-letter code for the mutation residue.

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alanine hGH, site 2 improved hGH, and octa-alanine site 2 improved hGH, respectively. For mutants with decreased site 1 affinity, crude ammonium sulfate precipitates (*11*) were applied to tandem Sephacryl-75 columns (Pharmacia) to yield 80−90% purity. Protein concentrations for these mutants were determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie blue stain as described (*10*).

Preparation of Site 2 hGH−Phage Libraries and Phage Sorting. Oligonucleotide-directed mutagenesis was used to construct four separate libraries using as a template the phagemid vector pH0753, which consists of the synthetic hGH gene fused to the carboxyl-terminal domain (codons 249−406) of M13 gene III (*14*). The parental DNA used for the random mutagenesis consisted of the hGH gene with five amino acid residues mutated to alanine: L45A, R64A, K172A, F176A, and R178A. The purpose of these alanine mutations was to diminish hormone binding to the hGHbp through site 1, thus allowing for the selection of hGH variants based on site 2 affinity. To abolish template-derived background and to suppress the possibility of codon bias to wild-type residues, all codons which were targets for randomization were mutated to stop codons (either TAA or TGA); single-stranded DNA that contained deoxyuracil was used for mutagenesis (*15*). The following oligonucleotiedes were used for random mutagenesis of target residues: 1092 (5′-GCTACAAATGCCTATGCA**NNSNNS**ACT**NNSNNS**-CTAAGT**NNS**CTATTCGATAACG-3′; for residues F1, P2, I4, P5, and R8 of the N-terminal library), 1093 5′-TAAGTC-GACTATTC**NNSNNS**GCTATG**NNSNNS**GCCCAT**NNS**CTT-CATCAGCTAGC-3′; for residues D11, N12, L15, R16, and R19 of the helix 1 library), 1094 (5′-CCAACAGCCTGGTC-**NNS**GGCGCCTCTGATTCG**NNS**GTGTAC**NNSNNS**CTG-AAGGACCTAGAG-3′; for residues Y103, N109, D112, and L113 of the loop/helix 3 library), and 1095 (5′-GTGTACGACCTGCTG-**NNSNNS**CTAGAG**NNS**GGGATCCAA**NNS**CTGATGGGG-**NNS**CTGGAAGAT-3′; for residues K115, D116, E119, T123, and R127 of the helix 3 library). The mutated DNA was electroporated into *E. coli* to give 7.3×10^7, 2.6×10^7, 3.8×10^7, and 1.3×10^7 independent transformants for the N-terminal, helix 1, loop/helix 3, and helix 3 libraries, respectively.

In preparation for phage sorting, a cysteine-locked, preformed dimer (S201C-L146C) of the hGHbp was coated on Maxisorp microtiter plates (NUNC, Roskilde, Denmark) at 1 μg/mL in 50 mM carbonate buffer, pH 9.6, overnight at 4 °C. Each of the libraries was sorted by adding approximately 5×10^{12} phage particles to the receptor-coated wells. Wells were washed extensively with 10 mM sodium phosphate/137 mM NaCl/2.7 mM KCl, pH 7.2 (PBS), with 0.05% Tween-20 (Sigma, St. Louis, MO). Phage particles were eluted with 0.1 M glycine, pH 2, and propagated as described (*13*, *14*). After four to six rounds of panning, individual selected clones were sequenced.

Kinetic and Equilibrium Binding Analysis of Selected hGH Clones. Variants of hGH selected from the site 2 libraries were expressed as free protein by transforming DNA into a nonsuppressor strain of *E. coli* (34B8; Genentech, Inc). Protein was isolated from the periplasmic space of *E. coli* by osmotic shock and ammonium sulfate precipitation as described (*10*). Protein concentrations were determined by densitometry of Coomassie-stained SDS−PAGE gels (*10*).

Association rates (k_{on}), dissociation rates (k_{off}), and equilibrium binding constants (K_d) were determined using surface plasmon resonance (*16*) on a BIAcore instrument. The cysteine-locked hGHbp was coupled to the sensor chip by random lysines to yield typically between 2500 and 3500 resonance units (RU). Dissociation rates were measured by passing 35 μL of either 2, 5, or 10 μM hGH in PBS with 0.05% Tween-20 at a flow rate of 20 μL/min over the sensor chip; after the injection, the decrease in RUs was monitored with time. Association rates were determined from the concentration dependence of the binding profiles for various hGH solutions. Typically, 1.25 μM solutions of ligand were serially diluted (2-fold) six times, and 35 μL was injected with flow rates of 20 μL/min. Sensor chips were regenerated as described (*11*). Binding profiles were analyzed using the provided software (BIAevaluation; Pharmacia Kinetics). Equilibrium dissociation constants were calculated by $K_d = k_{off}/k_{on}$. Kinetics of binding for site 1 decreased mutants was performed essentially as described above with the exception that the sensor chip was coupled with S102C hGHbp as previously described (*11*). Kinetic data from the BIAcore assay were analyzed by a one-exponential fit; however, the dissociation curves were sometimes bi- or multiphasic. We believe this effect was caused by minor proteolysis of Phe-1 on hGH which is involved in binding through site 2 (*3*); mass spectrometry of these variants revealed that approximately 10−20% of the expressed protein (depending on the variant) was missing Phe-1 due to an N-terminal proteolytic clip (data not shown).

Cell Proliferation Assays. The interleukin-3-dependent promyeloid cell line, FDC-P1, stably transfected with either the full-length or a truncated version, D351Stop, of the hGHR was used for cell proliferation assays as described (*17*). Cells were maintained in RPMI-1640 media with 10% fetal bovine serum (FBS) and 10 mM hGH. In preparation for the assay, cells were washed in PBS, resuspended in RPMI-1640 media with 1% FBS (fasting media), and fasted for 16−18 h. After fasting, cells were pelleted, washed with PBS, and resuspended to 1.5×10^5 cells/mL in hGH-free, RPMI-1640 media with 10% FBS. Cells were added to solutions containing respective concentrations of hGH or hGH mutants to give 20 000 cells/well in 200 μL total volume. Mixtures were incubated for 18−22 h, spiked with 1 μCi of [^{3}H]-thymidine for 4 h, harvested, and counted. The EC_{50} values for cell proliferation were virtually the same as those previously reported (*17*); the number of hGH receptors per cell was about 2-fold lower [~2000 and 200 hGH receptors per cell for full-length and D351Stop cells, respectively (data not shown)]. The lower receptor numbers per cell could have resulted from downregulation, as our cells were maintained in the presence of 10 nM hGH instead of IL-3 (*18*).

JAK2 Immunoprecipitation, Electrophoresis, and Immunoblotting. JAK2 precipitation from activated, lysed cells was performed essentially as described by Wang and Wood (*19*). Cells were maintained, fasted, pelleted, and resuspended in the fasting media to a density of 5×10^7 cells/mL. Cells (1 mL) were added to tubes containing a small volume of hGH to give the appropriate final hormone concentration. Mixtures were incubated for either 2 min (full-length hGHR) or 30 min (D351Stop) before the reaction was stopped with ice-cold PBS containing 1 mM Na_3VO_4. Pelleted cells were resuspended in 0.5 mL of ice-cold lysis buffer (20 mM Tris-

HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 1 mM $Na_4P_2O_7$, and 10 μg/mL aprotinin, pepstatin, and leupeptin), vortexed, and kept on ice for 15 min. Anti-JAK2 antiserum (5 μL; Upstate Biotechnology, Inc., Lake Placid, NY) was added to lysed cells, and tubes were rotated end over end for 1 h at 4 °C before the solution was clarified by centrifugation. Protein A−agarose beads (50 μL of 50% v/v in PBS; Boeringer Mannheim) were added to the supernatants, and solutions were incubated with rotation for 1 h at 4 °C. The beads were washed twice with ice-cold lysis buffer and once with 10 mM Tris-HCl/150 mM NaCl, boiled in 50 μL of 2× SDS sample buffer (Novex) with 0.1 M β-mercaptoethanol for 2 min, and electrophoresed on a 4−12% gradient Tris−Glycine SDS−PAGE (Novex). Gels were blotted to poly(vinyl difluoride) membranes (Novex) and probed with an anti-phosphotysosine antibody (4G10; Upstate Biotechnology, Inc.). Membranes were reprobed with the anti-JAK2 antiserum by first treating previously probed membranes with a 62.5 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol, 2% SDS solution for 30 min at 50 °C. Detection of probed membranes was performed using the enhanced chemiluminescence protocol (Amersham, Inc.), and films were analyzed by densitometry (Bio-Rad). Various film exposures were used to be certain that all points were within the linear range of detection.

RESULTS

Selection of High-Affinity hGH Site 2 Variants by Monovalent Phage Display. We used monovalent phage display (*12*, *13*) to construct an hGH variant with increased site 2 affinity as was previously done for improving site 1 affinity (*14*). hGH will not bind to the hGHbp through site 2 alone; thus, to begin the selections, it was necessary to construct a disulfide-locked dimeric form of the receptor (S201C-L146C) that could interact with the hormone at both site 1 and site 2. The receptor dimer was constructed on the basis of the X-ray crystal structure of the 1:2 hGH−hGHbp complex (*4*). However, this locked hGHbp dimer bound hGH so tightly ($K_d \ll 1$ pM) that we could not measure a dissociation constant (results not shown). Thus, before selecting for site 2 affinity-improved variants, we weakened site 1 affinity. This was achieved by introducing five mutations into site 1 (L45A/R64A/K172A/F176A/R178A); this hGH variant was then used as a template to introduce random mutations throughout the site 2 interface.

The site 2 receptor contact residues (*4*) were divided into four separate libraries so that all possible substitutions could be screened exhaustively (Figure 1). Codons to be randomized were first changed to stop codons in the template DNA to suppress the selection of wild-type protein sequences and to control against codon bias due to preferential annealing of the mutagenic oligonucleotides. The total number of transformants for the four libraries was 7.3×10^7, 2.6×10^7, 3.8×10^7, and 1.3×10^7 for the N-terminal, helix 1, loop/helix 3, and helix 3 libraries, respectively. Phage expressing the hGH site 2 libraries were sorted and propagated against the cysteine-locked hGHbp dimer (S201C-L146C) which was immobilized on plates. By the fourth and fifth rounds of selection, enrichments over binding of the starting hGH molecule (L45A/R64A/K172A/F176A/R178A) were ~10-fold for the helix 1 library and 2−4 fold for the N-terminal, loop/helix 3, and helix 3 libraries.

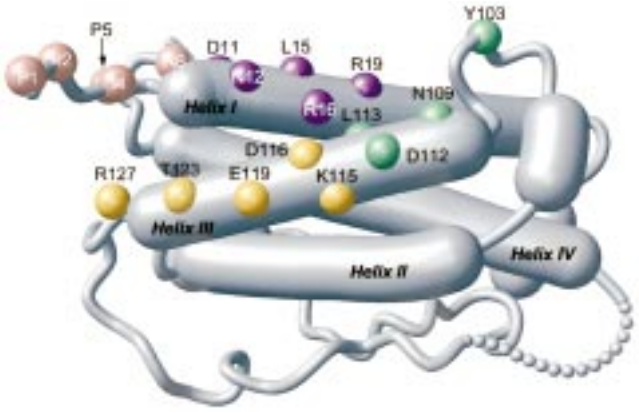

FIGURE 1: Site 2 residues on hGH targeted for randomization. Shown are the residues spanning helix 1 and helix 3 on hGH that were chosen for mutagenesis. Library 1 randomizes residues F1, P2, I4, P5, and R8 (light red); library 2 randomizes residues D11, N12, L15, R16, and R19 (purple); library 3 randomizes Y103, N109, D112, and L113 (green); and library 4 randomizes K115, D116, E119, T123, and R127 (yellow).

The sequences of phage clones isolated after three to five rounds of selection showed a clear consensus at most of the randomized sites (Figure 2). In the N-terminal library, residues 1 and 8 were dominated by large hydrophobic residues (either W, F, or Y) whereas the predominant mutations at positions 2, 4, and 5 were P2E, I4L, P5T, and P5R. Very strong selection was observed for D11E, N12G, and R19W from the helix 1 library. Little consensus was seen at position L15, and residue R16 was conserved as wild type. Most of the residues in the loop/helix 3 library showed no consensus with the exception of N109E. Most of the clones sequenced from the helix 3 library yielded the clone K115R/T123V where positions D116/E119/R127 were conserved as wild type.

On the basis of the sequence data, a number of selected clones from each of the libraries were chosen for affinity screening. Soluble protein was expressed and analyzed for binding to immobilized locked hGHbp dimer by surface plasmon resonance on the BIAcore (Pharmacia). In general, variants from the helix 1 and loop/helix 3 libraries yielded the most significant improvements in dissociation rate (Figure 3A). Compared to the off-rate for binding of hGH (L45A/R64A/K172A/F176A/R178A), the selectants that showed the most improvement in dissociation rate were D11E/N12G/L15A/R19W (5.5×) from the helix 1 library and Y103T/N109E/D112Y/L113T (3.5×) from the loop/helix 3 library. Only small changes in association rate were observed for all the mutants (Figure 3B). Thus, the clones that gave slower dissociation rates were generally the ones that yielded the most improvement in apparent dissociation constant, K_d (Figure 3C).

Combination of Site 2 Consensus Residues and Binding Analysis. The site 2 mutations that gave the most significant improvements in binding to the locked hGHbp dimer were incorporated into a single hGH mutant by site-directed mutagenesis. This combination mutant consisted of 14 mutations throughout the site 2 interface: P2E/I4L/P5R/R8W/D11E/N12G/L15A/R19W/Y103T/N109E/D112Y/L113T/K115R/T123V (designated site 2-improved hGH). To

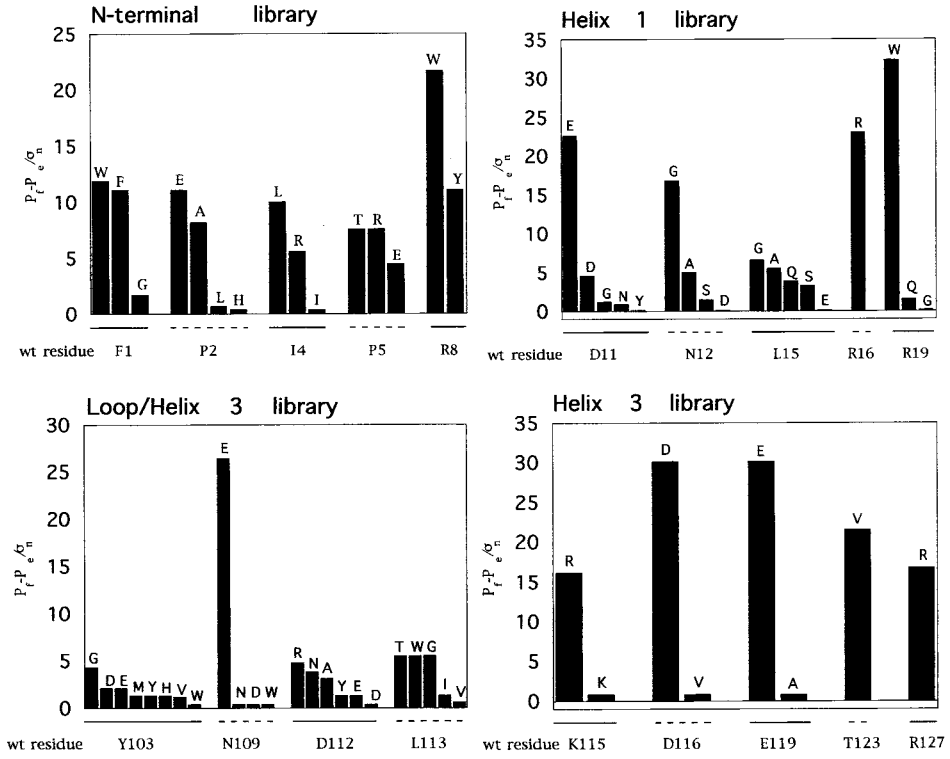

FIGURE 2: Consensus residues for sorting site 2 phage libraries. Shown are sequencing results after sorting rounds 3, 4, and 5. Data are represented as the likelihood of appearance for a particular amino acid as $(P_f - P_e)/\sigma_n$, where P_f is the fraction of residues found over all clones sequenced, P_e is the expected random frequency of appearance based on NNS codons, and $\sigma_n = [P_e(1 - P_e)/n]^{1/2}$. The number of sequences, n, represented are 34 for library 1, 35 for library 2, 31 for library 3, and 30 for library 4.

test for the total improvement achieved in receptor binding through site 2, these affinity-improving mutations were made in the background of the site 1 debilitating mutations L45A/R64A/K172A/F176A/R178A. Analysis by surface plasmon resonance showed that the site 2 combination mutant bound the locked hGHbp dimer approximately 38-fold tighter than wild type (Table 1). The effects on free energy of binding were reasonably additive because the product of the affinity improvements from the four chosen individual clones is predicted to produce a 45-fold increase in affinity for the locked hGHbp dimer. Such additivity has been seen for affinity improvements in site 1 as well (*14*).

To compare the bioactivity of the 40-fold affinity improvement in site 2 to site 1 affinity enhancement, a site 1 improved variant of hGH was produced that had a 50-fold increase in affinity for the hGHbp. This mutant, called octa-alanine hGH (H18A/Q22A/F25A/D26A/Q29A/E65A/K168A/E174A), was constructed by combining eight alanine mutations which individually improve affinity for the hGHbp (*11*). Both BIAcore and radioimmunoassays showed that the affinity of octa-alanine hGH for hGHbp was approximately 20 pM compared to about 1 nM for wild type (B. C. Cunningham, data not shown).

Cell Proliferation Assays for the Affinity-Improved hGH Variants. Transfection of the IL-3-dependent promyeloid cell line, FDC-P1, with the human growth hormone receptor (hGHR) allows these cells to proliferate when treated with hGH in the absence of IL-3 (*17*, *20*). Colosi et al. (*17*) have produced a receptor mutant (hGHR D351Stop) which expresses only 54 amino acids of the 350 amino acid intracellular domain, and this truncation is sufficient for JAK2 phosphorylation and cell proliferation. This variant receptor lacks the C-terminal domain involved in feedback inhibition by a tyrosine phosphatase. We used both the full-length and the D351Stop cell lines to investigate the ability of the affinity-improved hormones to stimulate cell proliferation.

As seen in Figure 4, neither the octa-alanine site 1, the site 2 affinity-improved, nor the combination site 1/site 2 affinity-improved hGH mutants showed any significant improvement in the EC_{50} for cell proliferation compared to the wild-type hGH response. All the mutants exhibited a full mitogenic dose−response curve and similar maximum responses. We also tested another site 1 variant of hGH that contained 15 substitutions which enhanced affinity by 400-fold compared to wild-type hGH (*14*). Even this variant exhibited virtually no improvement in the EC_{50} compared to wild-type hGH (70 versus 100 pM, respectively) (data not shown).

Previous studies have shown that hGH is internalized and degraded (*21*) and that the mitogenic response can be limited by ligand clearance from growth medium (*22*, *23*). To ensure that cell growth was not limited by ligand depletion at low hormone concentrations, we conducted the cell proliferation assay with the same cell number and hormone concentration but in a volume of 5 mL instead of the standard volume of 0.2 mL. The EC_{50} values were the same for both assay volumes, suggesting that ligand depletion does not significantly affect the EC_{50} (data not shown).

JAK2 Phosphorylation Assays for the Affinity-Improved hGH Variants. It has been shown that one of the initial events after dimerization of the hGH receptor is the phosphorylation of the intracellular tyrosine kinase, JAK2 (*6*). To test whether the high-affinity hGH variants could influence the levels of

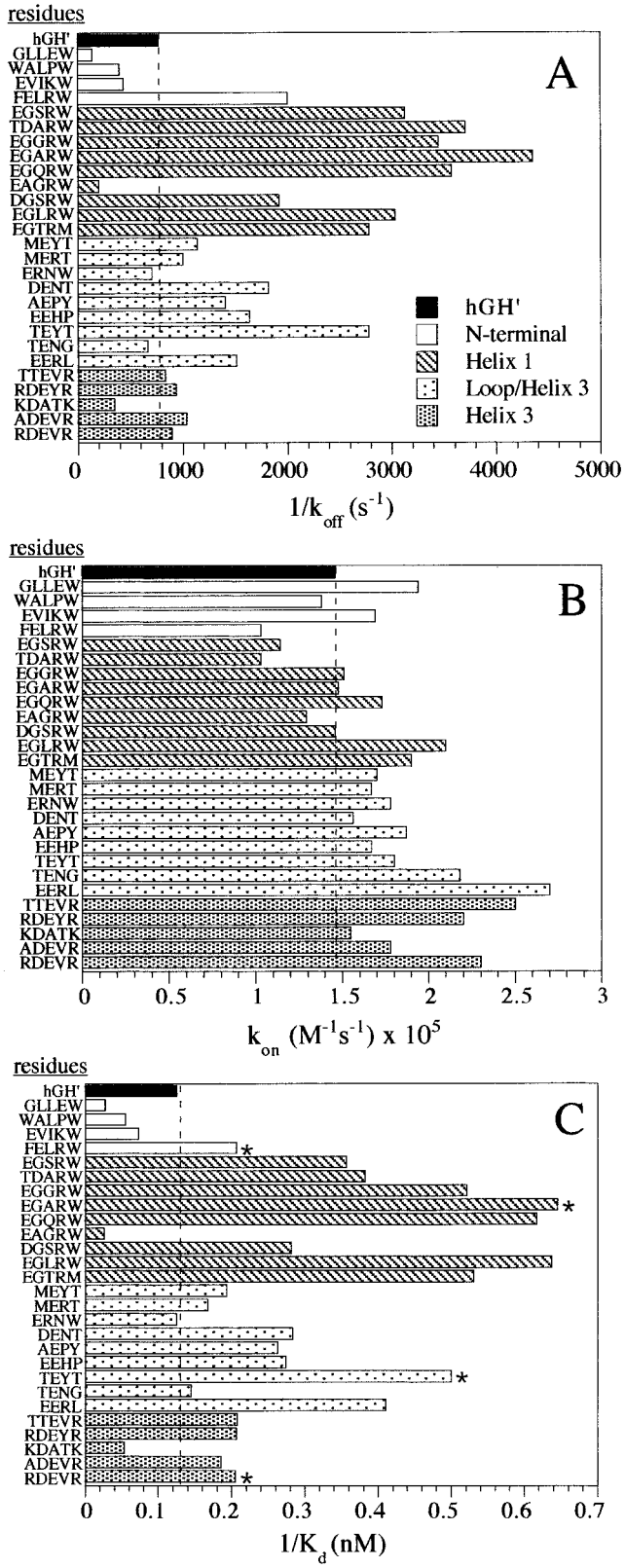

FIGURE 3: BIAcore analysis of selected clones after phage sorting. A number of clones from each library were selected after the third, fourth, and fifth rounds of phage sorting. Shown are the results for (A) dissociation rates, (B) association rates, and (C) relative affinity ($K_d = k_{off}/k_{on}$). The asterisk (*) represents clones that were chosen for combination into the site 2 affinity-improved mutant.

JAK2 phosphorylation, we used an immunoprecipitation and Western blot assay (*19*) to monitor JAK2 activation in FDC-P1 cells. JAK2 phosphorylation shows a bell-shaped dose−response with an EC_{50} value of 5−20 nM hGH and an IC_{50} value of about 4 μM (Figure 5). We measured JAK2 phosphorylation curves for each of the hGH mutants using both the hGHR and D351Stop cell lines and found no effect on the EC_{50} for JAK2 phosphorylation (Figure 6). Thus, increases in either site 1 or site 2 affinities of hGH do not enhance the bioactivity whether measured by early events such as JAK2 phosphorylation or late events like [^{3}H]-thymidine incorporation.

Cell Proliferation Assays for hGH Mutants with Decreased Affinity at Site 1. A series of alanine variants of hGH were produced to test how a systematic reduction in binding affinity correlates with potency in the cell-based assays. On the basis of previous studies (*11*), 12 single or multiple alanine mutants of hGH were produced with reduced affinities for hGHbp between 5- and 500-fold (Table 2). BIAcore kinetic analysis showed that the decrease in affinity was almost exclusively a result of increasing the off-rate (k_{off}) and not decreasing the on-rate (k_{on}). We measured the ability of these mutants to stimulate cell proliferation on both the full-length hGHR and the D351Stop FDC-P1 cell lines. A plot of the EC_{50} value versus the measured k_{off} (Figure 7) shows very little change in the EC_{50} for cell proliferation for those mutants with less than a 30-fold increase in k_{off}. There was generally a linear increase in EC_{50} with k_{off} once k_{off} was about 30-fold increased over wild-type hGH.

DISCUSSION

The focus of this investigation was to explore how hormone−receptor binding affinity relates to ability to transduce signal. The interaction between hGH and its receptor is an ideal system for this because of the extensive database of structural and functional information; a dimerization mechanism for receptor activation is well documented (*2*). Simple equilibrium models (*24*) have been developed for sequential dimerization of the hGH and EPO 1:2 hormone−receptor complexes (*8*, *9*). If hormone reaches equilibrium with receptor dimers on the cell surface, then at subsaturating concentrations it follows that more dimers would be formed with a high-affinity hormone variant than with a lower affinity variant at a similar concentration. Furthermore, if the measured response (either cell proliferation or intracellular kinase activation) were proportional to the number of receptor dimers at the cell surface, then we would expect to observe activity differences that would correlate with ligand−receptor affinity. Increased receptor affinity has previously been engineered into site 1 of hGH either by phage display (*13*) or by alanine mutation (*11*). Here, a strategy was developed whereby site 2 affinity could be increased. Hormones where both site 1 and site 2 affinities were increased were then used in bioactivity assays to test the model predictions.

Phage Display for hGH Site 2 Affinity Optimization. Phage display at two other protein−protein interfaces, the site 1 interface between hGH and the hGHbp (*14*) and the interface between heregulin and ErbB3 (*25*, *26*), has shown a strong correlation between residues conserved in phage selection and their importance to binding by alanine scanning. We find a similar correlation here. There are primarily five residues, F1, I4, R8, D116, and E119, throughout the hGH site 2 interface that have been shown to be important for

Table 1: Kinetic and Equilibrium Analysis of the hGH′ and the hGH′ Site 2 Affinity-Improved Variants for Binding to the Cysteine-Locked hGHbp Complex[a]

ligand	k_{off} (s^{-1}) × 10^{-4}	k_{on} (M^{-1} s^{-1}) × 10^{5}	K_d (nM)	K_d (hGH′)/K_d (mut)
hGH′	4.28 (±0.91)	0.71 (±0.07)	6.0 (±1.4)	
hGH′ site 2 affinity-improved	0.25 (±0.05)	1.60 (±0.30)	0.16 (±0.03)	38

[a] The hGH′ molecule is a variant of hGH with site 1 determinant residues mutated to alanine: L45A/R64A/K172A/F176A/R178A. The hGH′ site 2 affinity-improved molecule consists of the site 1 debilitating mutations in addition to the 14 site 2 affinity-improving mutations: P2E/14L/P5R/R8W/D11E/N12G/L15A/R19W/Y103T/N109E/D112Y/L113T/K115R/T123V.

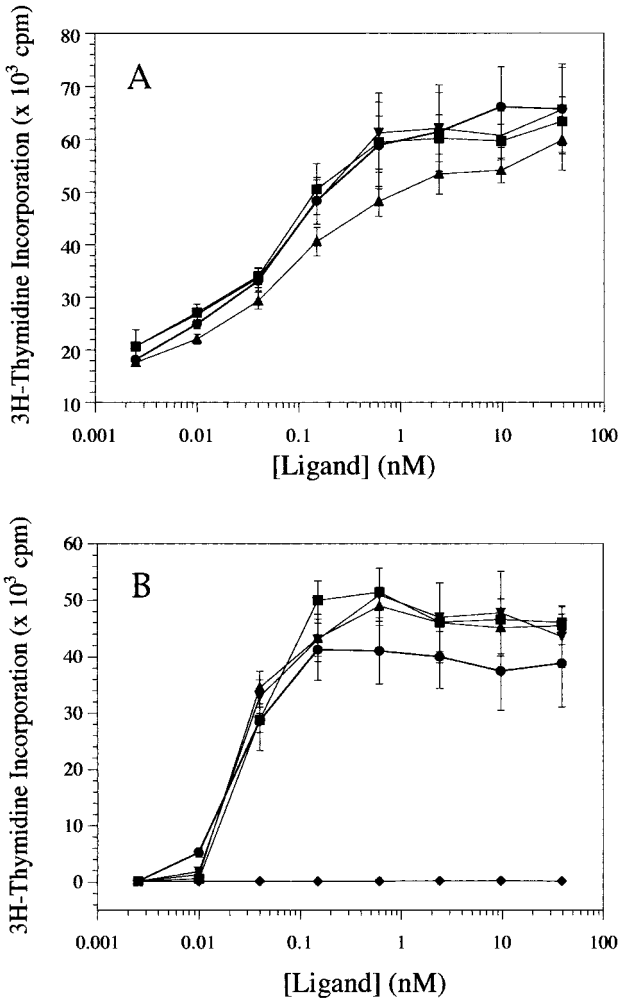

FIGURE 4: FDC-P1 cell proliferation for hGH affinity-improved variants. Shown are cell proliferation curves for both the full-length hGHR FDC-P1 cell line (A) and the D351Stop FDC-P1 cell line (B). Symbols represent the following hGH variants: (●) wild type, (■) site 1 affinity-improved, (▲) site 2 affinity-improved, (▼) site 1/site 2 affinity-improved, and (◆) G120R, which is an hGH mutant incapable of inducing receptor dimerization (*5*). Results are the average of duplicate experiments with each trial consisting of triplicate runs.

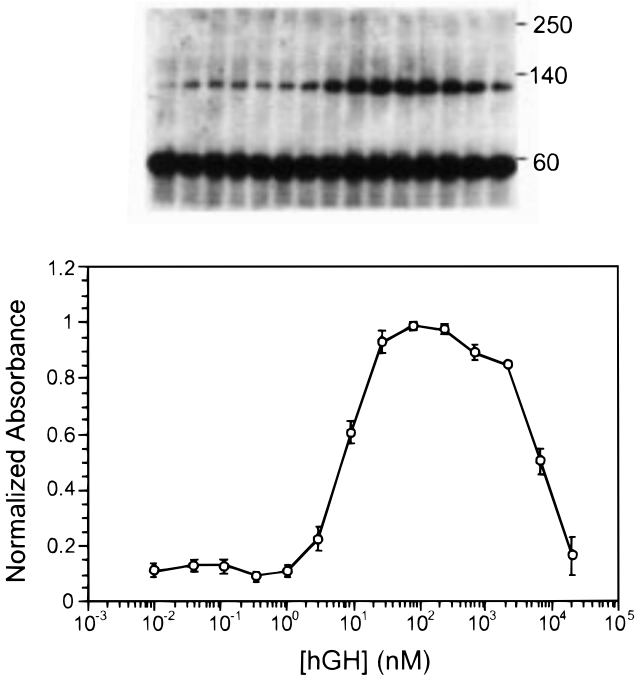

FIGURE 5: Typical JAK2 phosphorylation assay for hGH on hGHR FDC-P1 cells. Shown is a typical Western blot for the hGHR FDC-P1 cell line activated with hGHwt. Cells were incubated with the respective hGH concentration, lysed, immunoprecipitated, electrophoresed, blotted, and probed with an anti-phosphotyrosine antibody. The membranes were developed and scanned by densitometry; scanned gels were analyzed by volume analysis using the provided software (Bio-Rad).

binding the second hGHbp molecule (*3*); mutation of these residues to alanine causes a 5-, 60-, 3-, 6-, and 2-fold increase in EC_{50} for receptor dimerization, respectively. Generally, these residues were either retained as wild type or conservatively mutated following phage display (Figure 2). D116 and E119 were completely conserved, and the isosteric nature of side chains at F1, I4, and R8 was relatively conserved with the F1W, I4L, and R8W substitutions (Figure 2). In contrast, some of the more radical changes from wild type (P5T, P5R, N12G, and N109E) were at positions that were found to have relatively little importance for site 2 binding by alanine scanning. The greatest affinity improvements occurred at the functionality less important contact residues (such as N12, L15, and N109), and this was also seen in both the site 1 and heregulin/ErbB3 interfaces. The fact that the site 2 interface randomized here is somewhat smaller than the site 1 interface (850 and 1300 Å^2, respectively) may explain in part why the maximal affinity improvements were smaller at site 2 than site 1 [40-fold versus up to 400-fold, respectively (*14*)].

Affinity Improvements of hGH for Its Receptor Do Not Translate into Activity Improvements. Simple equilibrium models for sequential dimerization of the hGH receptor predict that the EC_{50} for the bell-shaped dose−response curve should relate to site 1 affinity, and the height of the curve should depend on site 2 affinity (*8*, *9*). On the basis of a model for receptor dimerization similar to one proposed by Ilondo et al. (*8*), it is estimated that an ~50-fold improvement in site 1 affinity should yield at least a 10-fold lower EC_{50} for cell proliferation if mitogenic response corresponds linearly to receptor dimerization/occupancy (*22*). However, none of the hGH variants that were improved in affinity for either site 1, site 2, or both showed a decrease in the EC_{50} for cell proliferation. Even a mutant that was 400-fold

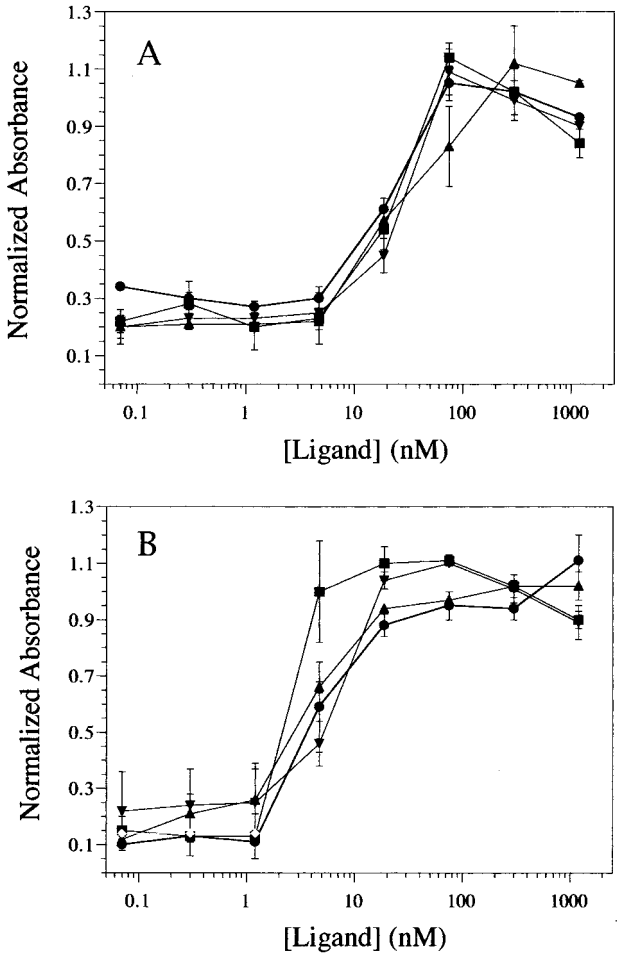

FIGURE 6: JAK2 phosphorylation assay for hGH affinity-improved variants. Shown are the results of densitometry analysis for each the hormones on both (A) FDC-P1 hGHR and (B) FDC-P1 D351Stop cell lines. See legend to Figure 4 for symbol definitions.

Table 2: Kinetic and Equilibrium Analysis of hGH Site 1 Affinity-Decreased Mutants Binding to Immobilized hGHbp[a]

ligand	k_{off} (s^{-1}) $\times 10^{-4}$	k_{on} (M^{-1} s^{-1}) $\times 10^{5}$	K_d (nM)	k_{off} (mut)/ k_{off} (wt)
hGH wt	5.4	3.5	1.6	1
D17A	31.3	3.0	10.4	5.8
L45A	26.4	2.8	9.4	4.9
D171A/Y164A	58.2	2.2	26.5	10.8
R64A	57.8	4.2	13.8	10.7
T175A	163	3.3	49	30.2
T175A/I179A	368	3.8	97	68.1
Y164A/R178A	406	2.4	169	75.2
L45A/R178A	549	2.5	220	101.7
D171A/T175A	496	2.5	198	91.9
R64A/T175A	1250	4.1	305	231
K172A/R178A	1975[b]	2.4	820	365
R64A/Y164A/T175A	2500[b]	1.8	1400	463

[a] The hGHbp mutant S210C was coupled to the sensor chip by standard methods (*11*). Each k_{off} value represents the average of two measurements. Typically, standard deviations are within 15% of the reported value. The on-rate value is a single measurement derived from the concentration dependence of association using four ligand injections. [b] The k_{off} values given for K172A/R178A and R64A/Y164A/T175A are approximations due to very rapid dissociation from immobilized S201C hGHbp.

enhanced in site 1 affinity (*14*) did not significantly change the EC_{50} values for cell proliferation (data not shown). Recently, Ballinger and co-workers (*26*) used phage display

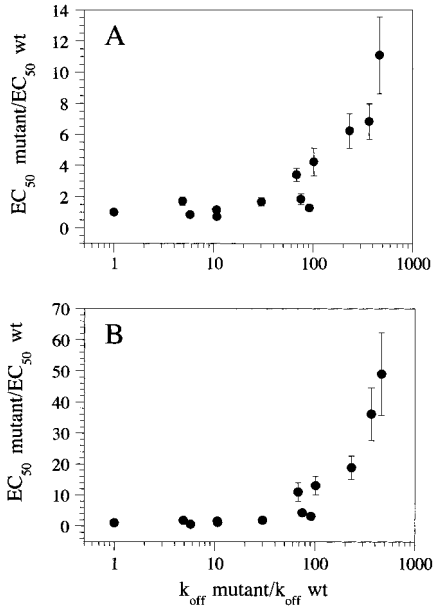

FIGURE 7: Cell proliferation for hGH site 1 affinity-decreased mutants. For each mutant, the k_{off}(mutant)/k_{off}(wild type) as measured by BIAcore analysis is shown plotted versus the EC_{50}(mutant)/EC_{50}(wild type) for cell proliferation on both (A) FDC-P1 hGHR and (B) FDC-P1 D351Stop cell lines. The values for the k_{off} ratio are shown in Table 2. EC_{50} values for the FDC-P1 assay were obtained using a four-parameter curve fit.

to produce a heregulin variant with 50-fold improved binding affinity for its receptor, ErbB3 (*26*, *27*). Similar to what we report here with hGH, this heregulin mutant showed no improvement in biopotency.

Several recent papers have highlighted the importance of downstream receptor events, such as receptor internalization, degradation, and recycling for determining hormone potency (*23*, *28*). For example, an epidermal growth factor (EGF) mutant with a 50-fold lower affinity relative to wild-type EGF has increased potency which is believed to be due to reduced ligand depletion and receptor downregulation (*23*). This result emphasizes the importance of hormone and receptor trafficking properties and the fact that receptor binding is only one event in a number of processes important for generation and maintenance of signal. We did not find that low-affinity hGH variants were increased in potency (see below).

JAK2 phosphorylation levels were very similar for both wild-type and affinity-improved hGH variants (Figure 6). It is interesting to note that, in general, the EC_{50} values for JAK2 phosphorylation (≈20 nM for the hGHR cells and ≈5 nM for the D351Stop cells) were significantly higher than the EC_{50} values measured for cell proliferation (≈0.1 nM for the hGHR cells and ≈0.02 nM for the D351Stop cells). This discrepancy might be caused by differences in total receptor numbers between the two assays. The typical receptor concentration was about 1000-fold lower in the cell proliferation assay (0.3 and 0.02 pM for wild-type and D351Stop receptors, respectively) compared to the JAK2 phosphorylation assay (0.2 and 0.02 nM, respectively). Another possibility is that only a small number of JAK2 molecules need to be activated to trigger the pathway to DNA synthesis and mitogenesis whereas a large fraction of the receptors needs to be occupied as dimers to yield detectable amounts of phosphorylated JAK2.

Small Decreases in Site 1 Affinity Do Not Affect the Biopotency of hGH. To investigate the effect of decreased hGH−hGHR affinity on cell proliferation, we made 12 hGH mutants that systematically decreased receptor affinity by increasing dissociation rate (Table 2). Figure 7 shows that, for hGH mutants where the dissociation rate was increased less than 30-fold, relatively little effect was observed in the EC_{50} for cell proliferation. For hGH mutants with more severely diminished k_{off} values at site 1, the effect on cell proliferation EC_{50} values is roughly linear. We conclude that the affinity of hGH for its receptor at site 1 is overmaximized for signaling ability.

Implications for the Receptor Homodimerization Mechanism. In the hGH sequential receptor dimerization mechanism, the initial site 1 binding event serves to capture ligand from solution; once localized to the surface, binding of the second receptor is greatly facilitated by both a high local concentration effect and a reduction in dimensionality at the membrane. From the data presented here, we can begin to estimate the effectiveness of a sequential dimerization mechanism.

After a hormone molecule diffuses in three dimensions to the first receptor, it then needs only to search in two dimensions to locate an unoccupied receptor. For a typical cell with a 20 μm diameter, 1000 receptors/cell, and assuming a 5 nm shell proximal to the membrane, the approximate concentration of occupied and unoccupied receptors will be 13 and 250 nM, respectively, at a hormone concentration $(1/10)K_d$. Site 2 affinity has been estimated to be approximately 0.5−5 nM in solution (3; K. Pearce, C. Schiffer, and J. Wells, unpublished results). Therefore, even at low concentration [such as $(1/10)K_d$], after site 1 binding, the concentration of hormone at the surface will be greater than site 2 affinity. This means that site 2 binding is highly favorable and that receptor dimers can form even at very low hormone concentrations. As our data with hGH show, the EC_{50} for cell proliferation is at least 10-fold lower than the K_d for site 1 binding.

In our model cells (20 μm diameter, 1000 receptors/cell), each receptor occupies about 1 μm^2 of surface area assuming a random distribution. When a wild-type hormone from solution forms a productive collision with a cell surface receptor, it can search approximately 20 μm^2 of surface area according to $\langle x^2 \rangle = Dt$, where $\langle x^2 \rangle$ is the mean free path, D is the lateral diffusion constant [10^{-10} cm^2/s (*29*)], and t is the residency time ($1/k_{off}$ = 2000 s). At subsaturating hormone levels, the area that an occupied receptor can search is about 20-fold larger than the area per unoccupied receptor. This ratio means that there is good probability that a dimeric complex will be formed during the lifetime of a 1:1 complex. Only when the off-rate approaches a 20-fold increase does the ratio of search area to area per receptor begin to approach a value of 1. This approximation roughly corresponds to that found experimentally (30−50-fold) when comparing bioactivity versus k_{off} (Figure 7). Therefore, in the hGH dimerization scheme, we conclude that the site 1 off-rate is maximized for biopotency. As our data show, increasing the lifetime of the 1:1 complex gives no signaling advantage, and in fact, signaling efficiency is rather unaffected by decreasing the lifetime of the 1:1 complex up to about 30-fold.

Some naturally occurring cells have a much higher receptor density [typically giving between 50 nM and 7 μM local concentrations (*30*, *31*)] than the hGHR-expressing cell line used in this study. Therefore, the affinity of hGH for its receptor may be even more overoptimized *in vivo* than in our model system. It remains to be seen whether or not a change in membrane fluidity or receptor density will systematically shift the potency inflection point to lower k_{off} values.

These results suggest how exquisite sensitivity can be achieved for a dimerization signaling mechanism. The cell can signal through receptor dimers even at hormone concentrations significantly below K_d, due to affinity enhancement that can be achieved by tethering a hormone to a cell surface (such as through site 1 binding). In addition, the dimerization mechanism may allow for several points of regulation, such as through changes in receptor density and membrane fluidity. Importantly, our results suggest that small molecule mimics of hGH might be effective signal transducers even if they have receptor binding affinities weaker than hGH.

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