Identification of Residues Outside the Two Binding Sites That Are Critical for Activation of the Lactogenic Activity of Human Growth Hormone*

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Human growth hormone (hGH) binds lactogenic or somatotrophic receptors, creating active heterotrimeric complexes. Comparison of hGH structures, either free or bound to a single lactogenic or somatotrophic receptor, shows binding is associated with structural changes. Changes in hGH structure are unique when binding either lactogenic or somatotrophic receptors and they influence the spatial arrangement of residues constituting the second receptor-binding site. Using site-directed mutagenesis, we identified a contiguous set of largely hydrophobic residues that forms a motif communicating between the two receptor-binding sites of hGH. The residues are external to the receptor-binding epitopes and were identified when their mutation reduced site 2 function without changing site 1 function. The motif includes Phe44, Leu93, Tyr160, Leu163, and Tyr164, located in two hydrophobic clusters between the receptor-binding sites. Their mutation to Glu disrupts hydrophobic interactions and reduces lactogenic activity between 4.7- and 85-fold with little effect on somatotrophic activity or spectroscopic properties. These differential effects indicate that loss of lactogenic activity is not a result of global mis-folding. We propose the loss of lactogenic activity results from disruption of specific hydrophobic clusters that disables the site 1 binding-induced structuring of the second receptor-binding site.

Human growth hormone (hGH)1 is a ligand of the subclass 1 of the hematopoietic receptor superfamily (1, 2) and is able to bind and activate both lactogenic and somatotrophic receptors (3). Administration of recombinant hGH is a treatment for some types of impaired stature and in elderly patients with reduced hGH blood concentrations (4). Extensive structural work has been performed on hGH including crystallographic and mutational studies, which make hGH an attractive target to study the mechanics of protein function. A thorough understanding of the mechanism by which hGH binds and activates receptors is required for the rational design of agonists and antagonists of hGH and other family members, most significantly prolactin and placental lactogen. hGH binds two receptors on unique surface topologies of the ligand, with receptor dimerization occurring in a sequential fashion (5, 6). The first binding occurs on a surface of hGH called site 1, followed by binding on site 2. X-ray crystallography structures are available for unbound hGH (Protein Data Bank code 1HGU) (7), hGH bound to one extracellular domain of the human lactogenic receptor (Protein Data Bank code 1BP3) (8), and hGH bound to either one (Protein Data Bank codes 1A22 and 1HWH) (9, 10), or two (Protein Data Bank codes 3HHR and 1HWG) (10, 11) extracellular domains of the somatotrophic receptor. The receptor-binding topologies of hGH (sites 1 and 2) are structurally distinct. The structural and functional binding epitopes of the hGH/hGH (extracellular domain of the hGH receptor) complex at site 1 (12) and site 2 (5) have been identified. Binding experiments with hGH and either the lactogenic or somatotrophic receptor describe extensively overlapping but non-identical receptor-binding sites on the ligand surface (13).

Comparison of the multiple crystal structures of hGH reveals that hGH undergoes structural changes induced by receptor binding at site 1 that are unique to the association with either lactogenic or somatotrophic receptors (Fig. 1) (14). These changes include helical structuring of residues 38–49 and 90–99, resulting in the formation of mini-helix 1 and a lengthening of helix 2, respectively. These large changes in helical content are coupled with a 15° shift of helix 3 relative to the axis of the 4-helix bundle. The lengthening of helix 2 must also reduce the mobility of helix 3 because site 1 binding reduces the non-helical residues connecting helices 2 and 3 from −13 to 3, positioning helix 3 in a more favorable position for site 2 binding. The articulation of helices 1 and 3 provides a groove that constitutes site 2 for either the lactogenic or somatotrophic receptor. Comparison of the structures of hGH bound to one extracellular domain of the somatotrophic receptor and hGH bound to two extracellular domains of the somatotrophic receptor suggests that these changes occur upon binding to the first receptor molecule.

Previous mutational analysis has shown that Phe44, located in the mini-helix region, is required for lactogenic activity, but not for somatotrophic activity (15). Although affecting biological activity, Phe44 is not located within the functional epitopes for either site 1 or site 2.

Ligand-binding assays have been useful in detecting steric hindrance within a single binding site. Because our studies focused on the mechanical interaction between the two binding sites, we used a cellular bioassay that measures the composite of all factors, both steric and mechanistic (16). In this way, we were able to discern an effect of hGH mutations that are not within the binding epitopes by their effect on activity.
We hypothesize that only the first receptor-binding site is present in apo-hGH and that a conformational change induced by lactogen receptor binding at site 1 transduces across the hGH molecule via a series of hydrophobic interactions, resulting in the structuring of a functional site 2. We used mutagenic scanning of the N terminus of helix 4 and hydrophobic residues in the 2–3 loop to identify a series of residues that form a hydrophobic motif functionally linking the first and second binding sites. It is this motif that articulates the conformation change. The ability to bind both lactogen and somatotrophic receptors not only allows us to distinguish the effects of lactogen receptor binding from those of somatotrophic receptor binding, but also gives us the ability to discern effects of the motif from effects of global protein disruption. Several studies (17, 18) have described the effects on biological and binding assays for ligands that have been mutated within the functional binding epitopes at site 1 or 2. In this work we identify a second category of hGH residues that may be useful in the development of antagonists include residues that inhibit the conformation required for binding of the second receptor molecule.

EXPERIMENTAL PROCEDURES

Plasmids and Bacterial Strains—An ϕI origin of replication was inserted into the pT7–7 plasmid (kindly provided by S. Tahor, Harvard Medical School, Boston, MA) generating the pT7–7ϕI plasmid. The positive strand was used for cloning, single-stranded DNA production, and expression of hGH. Escherichia coli strains DH5α, RZ1032, and BL21(DE3) were used for cloning, production of single-stranded DNA, and protein expression, respectively. This system was previously described (15, 19).

Expression, Purification, and Characterization of Recombinant hGHs—Proteins were expressed in BL21(DE3) cells and purified by DEAE chromatography (15, 19). Proteins were evaluated for size and purity by 15% SDS-PAGE under reducing and non-reducing conditions (20). Samples were prepared in 5% glycerol, 3% sodium SDS, 2% 2-mercaptoethanol, and a trace of pyronin Y, placed in boiling water for ~1 min, and 5–10 μg samples were placed on the gels. Gels contained sodium SDS without reducing reagent, were run at constant amperage, and were stained with Coomassie Brilliant Blue R. Proteins were evaluated for homogeneity and size by comparison with molecular weight markers. Protein folding was evaluated by absorption, fluorescence, and circular dichroism spectroscopy. Mutants constructed included F44E, V90E, L93E, R94F, P97E, Y105E, L157E, K158E, Y160E, L163A, L163E, L163F, and Y164E. Protein concentrations were measured by the bicinchoninic acid protein assay (21).

FDC-P1 Somatotrophic and Lactogenic Assays—FDC-P1 cells containing either the human prolactin or hGH receptor were a gift from Genentech Inc. (South San Francisco, CA). hGH dose-response curves were obtained as previously described (15, 16). Briefly, FDC-P1 cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 5 μg/ml gentamycin sulfate, and 1 nm wild-type hGH (22). One day before the assay, log-phase cells were collected and washed with non-supplemented RPMI 1640. Cells were washed and suspended in medium devoid of hGH and phenol red, but supplemented with 10% gelding horse serum. After 24 h, cells were collected and suspended in the same medium. Hormone stocks were diluted in this medium to the desired concentrations and added to 96-well plates in triplicate sets. Each well contained 15,000 FDC-P1 cells in a total volume of 100 μl. Plates were gently agitated and then incubated at 37 °C in a 5% CO2/95% air atmosphere for 48 h. Hormone-induced cell proliferation was assessed by a vital dye method by addition of 10 μl of Alamar Blue (Accumed International, West Lake, OH) per well, followed by a 4 h incubation. The oxidation-reduction of Alamar Blue was evaluated at 570 and 600 nm. These values were used to calculate the percent reduction of the dye, which is highly correlated with the number of cells (r2 > 0.99). The values obtained from dose-response studies were used to calculate ED50 values for the agonist phases by a four parameter fit method (23).

RESULTS

Protein Expression—Wild-type and all mutant hGHs were expressed and purified, yielding an average of 40 mg/liter of fermentation. All mutants, with the exception of L163E, co-migrated with wild-type protein on SDS-PAGE under both reducing and non-reducing conditions. Reducing conditions showed the proteins to be greater than 95% pure, whereas the non-reducing conditions suggested that all proteins were folded homogeneously, and were similar to wild-type hGH with only small traces of dimerized protein present. The SDS-polyacrylamide non-reducing gel of L163E hGH contained many bands, corresponding to variously folded and dimeric proteins (data not shown).

Protein Spectroscopy—UV absorption spectra were collected and corrected for background and light scatter. Spectra were normalized by dividing the absorbance at each wavelength by the value at 277 nm. All spectra were compared with that of wild-type hGH (Fig. 2). All mutants had similar curves, although five proteins varied significantly in their 250:280 nm ratio. These five proteins, L93E, R94F, L157E, L163E, and Y164E, showed increased 250:280 ratios of 42, 36, 34, and 35%, respectively. With the exception of L163E, all fluorescent spectra had similar emission spectra when normalized at 338 nm (Fig. 3). L163E had a reduction in quantum yield of 29% as
well as a secondary peak near 318 nm when compared with wild-type hGH. Only two other proteins had any significant variation in the spectra. V90E was red-shifted 4 nm, and R94F had an increased quantum yield of 18%. Circular dichroism spectra (Fig. 4) were typical of proteins with high helical content. Mutant proteins had molar ellipticity values at 222 nm within 10% of wild-type values except for V90E and L163E, which had 117.7 and 74.7% of wild-type values, respectively.

FDC-P1 Somatotrophic and Lactogenic Assays—Bioassays were performed at concentrations extending to 10,000 nM, a concentration at which both the agonist and antagonistic portions of the dose-response curve could be seen (Figs. 5 and 6). Results were normalized by identifying the wild-type hGH concentration providing the highest response and dividing the values for each dose of each protein evaluated in the assay by this number.

The coefficient of variation for the measurement of lactogenic ED_{50} values averaged 14.7%. We selected a considerably higher 2-fold cutoff value, a disproportionate decrease in lactogenic and somatotrophic activities and the curve shapes of the lactogenic bioassay to identify the residues included in this motif. We have conservatively selected residues for inclusion in this motif because we believe that fold-changes less than a 2-fold loss might be accounted for by biological variation in between assays. Protein aggregation was not present at hGH concentrations used in the bioassays, based on light scattering data at 350 nm (data not shown), which was performed at 2 μM, twice the highest concentration used in the bioassays.

F44E and Y164E hGHS had the greatest losses of activity in the FDC-P1 lactogenic assay relative to wild-type, 83.8- and 86.5-fold, respectively (Fig. 6 and Table I), and large decreases in lactogenic activity compared with relatively small changes in somatotrophic activity (Fig. 5 and Table I). Other hGH mutations that produced a significant loss in lactogenic activity included L93E and Y160E at 4.7- and 9.0-fold, respectively. These mutations had smaller effects on somatotrophic activities. Mutation of each of these four residues right-shifted the agonist phase of the dose-response curve, while not affecting the antagonist phase. Agonist activity requires both sites 1 and 2 to be functional, whereas antagonist activity requires only site 1 to be functional. Therefore, the data indicate that these mutations functionally uncouple site 1 from site 2. Based on these data, four residues were chosen for inclusion in the motif that propagates the conformation change from site 1 to site 2.

R94F hGH was not selected based on similar changes in both the lactogenic (2.5-fold) and somatotrophic (3.8-fold) bioassays. In both proliferation assays L163E, although promoting some proliferation, did not yield curves that could be analyzed for ED_{50} values.

Because Tyr^{164} is part of the structural binding epitopes (8), binding studies were performed to ensure that loss of biological activity wasn’t a result of a loss of binding at site 1. Wild-type and Tyr^{164} hGHS were placed in competitive I^{125} binding assay, and the relative K_{d} values were determined to be 0.047 and 0.070 nM, respectively.

Leu^{162} Mutants—Because Leu^{162} hGH shows a highly altered structure by spectroscopic studies and was inactive in both the FDC-P1 somatotrophic and lactogenic bioassays, it was determined that the residue was critical to the global
structure of the protein. To resolve the role of Leu163, more conservative amino acid substitutions, alanine and phenylalanine, were prepared. Both of these mutant proteins had full activity in the FDC-P1 somatotrophic assay. In the FDC-P1 lactogenic assay they showed a 3.5- and a 9.0-fold loss with alanine and phenylalanine, respectively (Table II). Therefore, this residue was also included in the motif that functionally couples site 1 to site 2.

**FIG. 6.** FDC-P1 human lactogenic bioassay. Hormone doses were prepared and administered as described under “Experimental Procedures.” Data are representative of two or more experiments. All proteins were evaluated in the same experiment. Each data point represents the average from triplicate wells. The maximum responses were normalized to the wild-type hGH maximal response. The large panel contains the dose response curves for mutants that had reduced lactogenic ED_{50} values. The inset contains dose response curves for all proteins tested. The average coefficient of variation for all data points was 2.03% with a range between 0 and 5.8%.

**TABLE I**

| FDC-P1 lactogenic assay | FDC-P1 somatotrophic assay |
|-------------------------|---------------------------|
|  | ED_{50} | Relative fold loss of activity | ED_{50} | Relative fold loss of activity |
| WT hGH | 1.76 | 1.00 | 0.0712 | 1.00 |
| F44E | 147 | 83.8 | 0.576 | 8.09 |
| V90E | 2.68 | 1.52 | 0.098 | 1.38 |
| L93E | 8.22 | 4.86 | 0.234 | 3.29 |
| R94F | 4.4 | 2.50 | 0.272 | 3.82 |
| F97E | 2.16 | 1.23 | 0.108 | 1.52 |
| Y103E | 1.98 | 1.13 | 0.107 | 1.52 |
| L157E | 1.83 | 1.04 | 0.107 | 1.52 |
| K158E | 2.15 | 1.22 | 0.116 | 1.63 |
| L160E | 15.8 | 9.00 | 0.186 | 2.62 |
| L163E | *** | *** | *** | *** |
| Y164E | 152.0 | 86.5 | 0.151 | 2.12 |

a *** values could not be calculated.

**FIG. 7.** Hydrophobic packing of hGH in the unbound form (left, Protein Data Bank code 1HGU) (7) and the 1.1 Å hGH/hPRL receptor complex (right, Protein Data Bank code 1BP3) (8). hGH is pictured in both panels as a ribbon structure in dark blue. The light blue represents the van der Waals space occupied by the entire molecule. The motif residues are represented as van der Waals space fill in solid red (with the non-motif residue of Y103) and highlight the alteration of packing in the interior of hGH due to lactogenic receptor binding at site 1. Distance measurements between residues of the motif decrease by between 5 and 11 Å when comparing hGHs either not bound or bound to the extracellular domain of a lactogenic receptor.

The second hydrophobic cluster contains Leu163, Val90, Leu93, Val96, and Phe97. Although each residue in the cluster was mutated to glutamic acid, only Leu163 and Leu93 reduced lactogenic activity. Using the crystal structure of hGH bound to one molecule of the lactogenic receptor (8), distances were measured from the two closest atoms in each residue pair, and a correlation between distance and loss of lactogenic activity was observed. Phe44 to Tyr164 and Phe44 to Tyr160 were closest at 3.287 and 3.282 Å, respectively, Leu163 and Leu93 were next closest at 4.285 Å, whereas other residues in the

**FIG. 7.** Hydrophobic packing of hGH in the unbound form (left, Protein Data Bank code 1HGU) (7) and the 1.1 Å hGH/hPRL receptor complex (right, Protein Data Bank code 1BP3) (8). hGH is pictured in both panels as a ribbon structure in dark blue. The light blue represents the van der Waals space occupied by the entire molecule. The motif residues are represented as van der Waals space fill in solid red (with the non-motif residue of Y103) and highlight the alteration of packing in the interior of hGH due to lactogenic receptor binding at site 1. Distance measurements between residues of the motif decrease by between 5 and 11 Å when comparing hGHs either not bound or bound to the extracellular domain of a lactogenic receptor.

The residues identified to be biologically relevant by mutagenesis experiments are rearranged to form a more tightly packed structure upon binding to the lactogenic receptor (Fig. 7, Protein Data Bank codes 1HGU and 1BP3) (7, 8). Large changes include a structuring of mini-helix 1, an extension of helix 2 by structuring of the 2–3 loop, and a rotation of helix 3 with respect to helix 1. The mechanism proposed to drive the conformational change begins with Phe44. The lactogenic receptor binding at site 1 constrains Phe44 by promoting the formation of mini-helix 1, inserting Phe44 between Tyr160 and Tyr164 in the N terminus of helix 4. Phe44 insertion lengthens and subtly unwinds helix 4 relative to the helical axis. Helix 4 rotation is constrained C terminus to the disulfide bond at Cys165. Leu163 extends ~180° away from the hydrophobic clus-
hydrophobic clusters had distances greater than 7 Å. Distances were measured from the two closest atoms in each residue pair. Distances greater than 4.5 Å provide sufficient space for water, thus diminishing the disruption of the hydrophobic packing by the glutamic acid mutation. Therefore, residues that most closely articulate in these hydrophobic cores provide the greatest functional disruption when mutated.

L163E hGH was the only mutant whose properties varied significantly in all three types of spectroscopy, electrophoresis, and biological activity, indicating that the mutation had altered the global folding of the protein. Accordingly, the somatotrophic assay also revealed a severe decrease in activity. The ED50 values could not be calculated for L163E hGH for either the lactogenic or somatotrophic assays. Interestingly, the somatotrophic assay curve showed several peaks and did manage to achieve 66% of the maximum response. The corruption of hydrophobic packing by replacing Leu163 with glutamic acid demonstrates the importance of packing in the interior of the protein for either lactogenic or somatotrophic activity. Loss of packing by replacement with a hydrophilic Glu resulted in a protein that assumes multiple conformations (as demonstrated by non-reducing SDS-PAGE), resulting in a protein that contains multiple transient folding states.

The data for Leu163 shows that mutation to a glutamic acid created large disturbances in the structure of hGH and emphasizes the centrality of this hydrophobic residue for both protein stability and function. Unfortunately, due to the global consequences of this mutation, its membership in the hydrophobic motif that propagates a conformation change could not be either supported or dismissed based on this mutation alone. To further investigate this critical residue, the additional mutations (L163A and L163F) were prepared. Both of these mutations alter the steric properties at Leu163 without negating the hydrophobicity. This alteration in side chain affects the packing of the closely articulating residues of the second hydrophobic core. L163A had a modest 3.5-fold loss in lactogenic activity. This may be the result of a shorter side chain (loss of three carbons) offering less surface area for hydrophobic packing and may no longer be close enough to Leu163 to exert as strong of an effect. L163F had a loss of lactogenic activity of 9.0-fold and a somatotrophic loss of activity of 3.3-fold. This leucine to phenylalanine mutation increased the bulk (addition of three carbons) and likely prevented tight packing in the second hydrophobic cluster, or resulted in poor alignment of the helix 2 extension (residues 90–99). These two mutations provide evidence for including Leu163 in the lactogenic motif, as well as to support the argument for its centrality in maintaining the overall structure of hGH.

Many of the mutations studied in this work are located in the interior of hGH, making it important to establish that the mutants were folded correctly. This was verified in several ways. Full biological activity in the somatotrophic assays indicated that the structure had not been globally changed and that any local change only affected lactogenic activity. The various spectroscopic methods yielded more detailed information regarding the fold of the mutant proteins. Circular dichroism indicated that the helical structure was intact. The fluorescent spectra monitored the environment of the single tryptophan located in the hydrophobic core of the 4-helix bundle. This data indicated that the hydrophobic interior of the protein had not been disturbed. The 250:280 ratios in the UV spectra are important for evaluation of disulfide bond formation, as disulfide bonds absorb at 250 nm (whereas free cysteines do not). The absorbance of a disulfide bond at 250 nm is highly dependent on bond angle and tension (25, 26). Therefore, the 250:280 nm ratio is a measure of disulfide bond characteristics normalized to total protein concentration. The spectra indicate that although several of the mutants have disulfide bonds that are strained, all of the disulfide bonds are intact.

R94F hGH serves as an interesting example. The large change in the 250/280 ratio indicates that the two disulfide bonds in R94F hGH are as strained as any of the mutations included within the motif. This single mutation only results in a 2.5- and 3.8-fold loss of lactogenic and somatotrophic activities, respectively, indicative of only modest structural disturbances. Therefore, the strains in disulfide bonds, although small, produce a large change in the 250 nm absorbance and are a sensitive probe for small mutation-induced structural changes.

The shape of the dose-response curves from the FDC-P1 lactogenic bioassays are unlike typical bell-shaped dose-response curves. Several other papers have described the effects on biological and binding assays for hGH mutated within site 1 or site 2 (17, 18). Hormones that activate via receptor dimerization are known to have bell-shaped dose-response curves. Mutations within site 1 that alter binding affinities either right or left shift both the agonist and the antagonist phases of the curve. This occurs because site 1 binding is involved in both the agonist and antagonist phases of the dose-response curves. Mutations in site 2 are indicated by modulations in the maximal biological response.

The dose-response curves seen for the exosite mutations show a shift in the agonist phase without a concurrent and proportional shift in the antagonist phase. The maximal biological activity is difficult to discern in some cases, as the antagonist phase is reached before the agonist phase is complete. Because the antagonist phase represents a population of receptors that are predominantly in a 1:1 complex with hGH, the ID50 is representative of site 1 binding affinity. Therefore, a shift in the ED50 without a shift in the ID50 indicates that a loss of activity is not due to binding at site 1 (16). These curve shapes are consistent with the notion of an induced-fit model, where site 1 is always available for binding, but where site 2 requires binding at site 1 to induce the conformation change required to create site 2.

Current theories for the mechanism of the sequential binding include a differential affinity model and a model in which free energy is provided through the stem-stem interactions of the two receptor molecules (17, 27). These models are not exclusive, and both play a role in receptor dimerization. We propose that the mechanism of the induced-fit model relies on a contiguous hydrophobic motif that propagates a site 1 binding-induced conformational change that orders and activates site 2. Such a model would complement the existing models to achieve a more complete mechanism of action. To conclude, we have found a unique motif that is required for the lactogenic activity of hGH but that is largely independent of somatotrophic action. Additionally, the concept of this type of motif may also be relevant in other ligand-receptor interactions among the proteins in the hGH superfamily and therefore be useful in the development of antagonists.

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