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Human growth hormone site 2 lactogenic activity requires a distant tyrosine164

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Abstract Comparison of crystallographic structures of human growth hormone, either bound to the prolactin receptor or free of receptors, reveals that human growth hormone binding to the prolactin receptor at site 1 is associated with a structural change in human growth hormone that influences the organization of residues which constitute site 2. We have identified Tyr^{164} as a residue that is critical for the propagation of this structural rearrangement. Tyr^{164} is a structural epitope for site 1 and is distal to site 2. Mutation of Tyr^{164} to glutamic acid (Y164E) does not affect the somatotrophic activity, absorption or fluorescence spectra or binding to the human prolactin receptor when compared to wild-type human growth hormone, indicating the subtle effects of the mutation. Lactogenic assays using extended concentrations of Y164E human growth hormone produce dose-response curves that are characterized by a right-shifted agonist phase and an unchanged antagonist phase when compared to wild-type human growth hormone. These results indicate that Tyr^{164} is required for the lactogenic activity of human growth hormone and that mutation to glutamic acid disrupts the lactogenic function of site 2.

1. Introduction

Human growth hormone (hGH) is a ligand of the subclass 1 of the hematopoietic receptor superfamily [1] and is able to bind and activate both somatotrophic and lactogenic receptors [2]. hGH binds two receptors in a sequential order on two distinct faces of the ligand (sites 1 and 2) [3]. hGH is an important recombinant protein marketed for treatment of some types of impaired stature and is recognized as having beneficial effects in elderly patients with reduced hGH blood concentrations [4].

X-ray crystallographic structures are available for receptor-free hGH (Protein Data Base number 1HGU), hGH bound to one extracellular domain of the human prolactin receptor [5] and hGH bound to either one (PDB number 1A22 and 1HWH)[6,7] or two (PDB number 3HHR and 1HWG)[7,8] extracellular domains of the hGH receptor. Comparison of these structures reveals that hGH undergoes structural changes associated with receptor binding and that are unique to binding either GH or prolactin receptors. From the evidence available, the structural changes in hGH are coincident with binding the first receptor at site 1 of hGH. The mechanism by which site 1 binding initiates these conformation changes and their functional importance are unknown.

Mutagenic studies offer a method to identify the residues which are functionally important [9]. Mutagenesis may identify members of the motif that transmit binding-induced structural changes to portions of the protein which are distal to site 1. Mutation of residues within a motif which propagates a conformation change may alter the conformation change and change dose-response curves in a predictable fashion. Such changes may be unique from those observed by mutations within the functional epitopes of sites 1 or 2.

Several papers have described the effects on biological and binding assays for ligands that have been mutated within the functional epitopes contained within sites 1 or 2 [10,11]. Hormones that stimulate target cells through receptor dimerization mechanisms display dose-response curves in biological assays that are bell-shaped where ligands function as agonists at low concentrations and antagonists at higher concentrations. The characteristics of extended dose-response curves differentiate the effects of mutations within sites 1 and 2. Mutations of the functional epitopes within site 1 either increase or decrease the ligand receptor affinity at this site and shift both agonist and antagonist phases of the dose-response curve to the left or right, respectively. In contrast, mutations within the functional epitopes of site 2 are reflected in modulation of the maximal biological activity. Mutations outside the functional epitopes of sites 1 or 2 may or may not affect dose-response curves. Interpretation of the effects of mutations which lay outside of the functional epitopes of sites 1 or 2 have not been described for biological or binding assays.

We have produced mutations in hGH that are either within (G120R) or external (Y164E) to the functional epitopes of site 2. The data from biological and binding studies demonstrate a previously unrecognized effect on dose-response curves which supports the hypothesis that selected residues distal from the second receptor binding site affect the ability of site 2 of hGH to activate the human prolactin receptor.

2. Materials and methods

2.1. Plasmids and bacterial strains

A f_1 origin of replication was inserted at a unique *Cla* I site in pT7-7 (kindly provided by S. Tabor, Harvard Medial School, Boston, MA, USA). The positive strand pT7-7 phagemid was used for cloning, production of ssDNA and expression of hGH. *Escherichia coli* strains DH5α, RZ1032 (dut^- and ung^-) and BL21(DE3) were used for cloning, production of uradin-substituted ssDNA and protein expression, respectively. The pT7-7f(+) phagemid for the expression of wild-type hGH has previously been described [12,13].

2.2. Site-directed mutagenesis

In vitro mutagenesis was performed by the method of Kunkel [14]. Primers were designed to produce the desired mutations and either add or delete a translationally silent restriction endonuclease site to allow rapid selection of potential mutants. Clones identified by restriction digests were completely sequenced to confirm the presence of the desired mutation(s).

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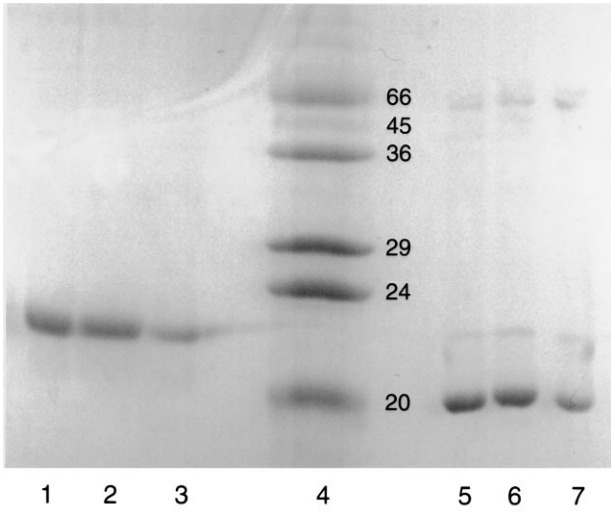

Fig. 1. 15% SDS-PAGE of recombinant hGHs. Wild-type (lanes 1 and 5), G120R (lanes 2 and 6) and Y164E (lanes 3 and 7) hGHs were prepared in the presence (lanes 1–3) or absence (lanes 5–7) of 1% 2-mercaptoethanol. Lane 4: reduced molecular weight markers in kDa. Fig. 1 is a digital image of the Coomassie-stained gel.

2.3. Expression, purification and characterization of hGHs

Purified phagemids containing wild-type, G120R or Y164E hGH DNAs were transformed into BL21(DE3) cells and expressed and purified as previously described [12,13]. Proteins were evaluated for size and purity by 15% SDS-PAGE under non-reducing or reducing conditions. Absorption and fluorescence spectra were collected at 20°C in 10 mM Tris pH 8.2, 150 mM NaCl.

2.4. FDC-P1 lactogenic assay

FDC-P1 cells containing the human prolactin receptor were a gift from Genentech (South San Francisco, CA, USA). Cells were maintained in RPMI 1640 containing 10 μM 2-mercaptoethanol, 1 nM wild-type hGH and 10% fetal calf serum [15]. Log phase cells were collected and washed three times with non-supplemented RPMI 1640. Washed cells were suspended in media devoid of wild-type hGH and phenol red but supplemented with 10% horse serum. Cells were maintained under these conditions for 24 h immediately prior to the assay. Recombinant hGH stock concentrations were determined by the bicinchoninic acid/copper sulfate assay [16]. Hormone stocks were diluted with phenol red-free media to the desired concentrations and added to 96 well plates in triplicate wells. 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% CO_2/95% air atmosphere for 48 h. Hormone-induced proliferation of the cells was assessed by a vital dye method with the addition of 10 μl of Alamar blue (Accumed International, West Lake, OH, USA) 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 percentage reduction of the dye, which is highly correlated with the cell number ($r^2 > 0.99$). The values obtained from dose-response studies were used to calculate ED_{50}s for the agonist phases by a four parameter fit method [17]. ID_{50}s for the antagonist phases were estimated from plots of the dose-response curves. In instances where the antagonist phase of the dose-response curve reached the x-axis, values determined by the four parameter fit method and graphical estimation were similar. Further extension of the dose-response curves were not possible due to the limited solubility of hGH.

2.5. FDC-P1 somatotrophic assay

Somatotrophic activities of each hormone preparation were determined with FDC-P1 cells transfected with the hGH receptor [18] as described by Peterson and Brooks [12]. These cells were a gift from Genentech (South San Francisco, CA, USA).

2.6. Lactogen receptor binding assays

hGH was iodinated using Iodogen (Pierce Chemical, Rockford, IL, USA) and carrier-free [^{125}I]iodine to a specific activity of 45 μCi/μg. Binding reactions contained membranes from 2×10^6 cells in 700 μl of RPMI 1640 medium supplemented with 25 mM HEPES at pH 7.4, 0.5% bovine albumin, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, approximately 1 ng of [^{125}I]hGH and various concentration of recombinant hGHs. Assays were supplemented with 1 mM $ZnSO_4$. Incubations were run for approximately 20 h at room temperature. Membranes were collected by centrifugation and membrane-associated [^{125}I]hGH was measured. Data from competition studies were used to calculate relative affinities by the method of Scatchard [19].

3. Results

3.1. Characterization of proteins

DNA sequence analysis of the pT7-7 phagemids showed sequences to be identical to those previously reported for hGH [20] or to contain the changes directed by mutagenesis. Yields of the purified proteins were greater than 20 mg/l fermentation. SDS-containing gel electrophoresis performed under reducing conditions (Fig. 1) revealed each hGH to be greater than 95% pure and have a molecular weight similar to hGH isolated from pituitaries. Electrophoresis in the absence of 2-mercaptoethanol showed that the proteins ran with a similar M_r, indicating that the proteins folded with the same pattern of disulfide bond which produced molecules with similar hydrodynamic radii [13].

Absorption spectra of Y164E and wild-type hGH overlay each other (Fig. 2), indicating that the tertiary structures of these proteins are very similar. In contrast, the spectrum of G120R hGH showed an increased absorption in the 250 and 340 nm regions. We interpret these results to indicate that the G120R mutation increases the aggregation of this protein at FM concentrations. The fluorescence spectra of the three proteins were similar, indicating that the environment of the the Trp and Tyr residues were unchanged by either of these mutations (Fig. 3).

In two trials, the somatotrophic activities of wild-type and Y164E hGHs produced similar ED_{50}s of 0.36 and 0.42 nM, respectively. G120R hGH displayed a somatotrophic ED_{50} of 2.31 nM. Both wild-type and Y164E hGHs had equivalent maximal activities, but G120R hGH displayed approximately half the maximal activity of wild-type hGH. These results are

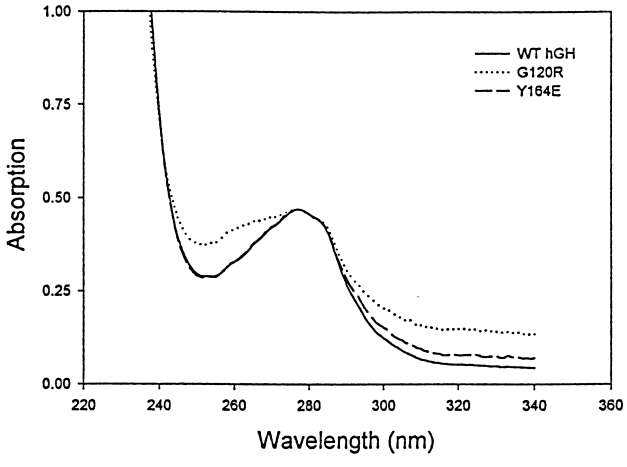

Fig. 2. Absorption spectra of recombinant hGHs. Wild-type, G120R and Y164E hGHs were prepared at 20 FM in 10 mM Tris pH 8.2, 150 mM NaCl. Spectra were collected at 20°C.

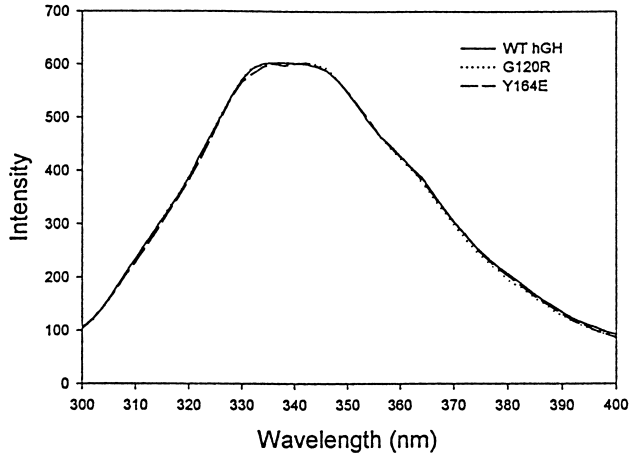

Fig. 3. Fluorescence spectra of recombinant hGHs. Wild-type, G120R and Y164E hGHs were prepared at 18 FM in 10 mM Tris pH 8.2, 150 mM NaCl. Spectra were collected at 20°C with an excitation wavelength of 280 nm.

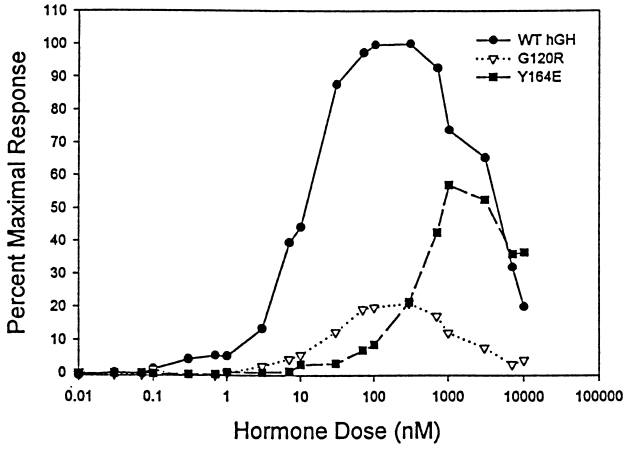

Fig. 4. FDC-P1 cells transfected with the human prolactin receptor and stimulated by recombinant hGHs. FDC-P1 cells were assayed as described in Section 2 and the relative hGH-induced increase in cell number was followed with a vital dye method. All three curves were run in the same experiment.

consistent with previous reports in the literature which describe Gly^{120} as a functional epitope of site 2 [6,15].

3.2. Activation of the lactogenic actions by hGH

Wild-type, G120R and Y164E hGHs were used to stimulate the growth of FDC-P1 cells that were transfected with the human prolactin receptor. Extended dose-response curves were performed to describe both the agonist and antagonist phases. FDC-P1 lactogenic assays displayed an average coefficient of variation of 28%. Based on our experience with this assay, a greater than 2–3-fold change in agonist or antagonist response was considered to be a significant change.

The wild-type hGH concentration required to elicit a half maximal agonist response (ED_{50}) was 12.1 nM (Fig. 4, Table 1), with the concentration required to antagonize the maximal response by 50% (ID_{50}) was $\approx$5750 nM.

Mutation of Gly^{120} to arginine did not significantly effect either the ED_{50} (19.5 nM) or ID_{50} ($\approx$3000 nM) of hGH. But the maximal response was reduced to 21% of that induced by wild-type hGH.

In contrast, the concentration of Y164E hGH required to induce the agonist phase of the dose response curves was 28-fold greater (ED_{50} = 338.8 nM) than that for wild-type hGH, while the ID_{50} ($\approx$10000 nM) was not changed by this mutation, remaining within 2-fold of the ID_{50} for wild-type hGH. The maximal response was reduced to 72% of the value for wild-type hGH. The ratio of ID_{50}/ED_{50} for wild-type hGH was 475, while the ratio for Y164E hGH had narrowed to 29.

The shapes and positions of the bell-shaped dose-response curves were affected by mutations. Wild-type and G120R hGHs had superimposed dose-response curves when they were normalized for the maximal response. The ED_{50} and ID_{50} for these two proteins differed by several hundred-fold. Y164E hGH displayed a dose-response curve where the ED_{50} and ID_{50} were more closely spaced differing by only a 29-fold. The agonist phase of the dose-response curve was shifted right from wild-type hGH, but the antagonist phases were coincident.

3.3. Prolactin receptor binding by hGH mutants

Competitive binding studies were performed with ^{125}I-labelled wild-type hGH and increasing concentrations of each hGH. Scatchard analysis of wild-type, G120R or Y164E hGH competitions revealed similar affinities, between 0.047 and 0.070 nM (Table 1). These competition studies represent measurements of the relative affinities of the competing protein for the lactogenic receptors.

4. Discussion

Tyr^{164} is distal to site 2 of hGH and has a pattern of agonist and antagonist activities that are distinct from both wild-type and G120R hGHs. The effects of the Y164E mutation are to right shift the agonist phase of the dose-response curve but not alter the antagonist phase. To date, we have observed similar changes in the dose-response curves of three other mutations that are external to both sites 1 and 2 (Duda, Peterson and Brooks, in preparation)

The ED_{50} of dose-response curves represent the final cellular response to increasing hormone concentrations. This response is determined by numerous coupled and complex processes including the relative affinities of sites 1 and 2 for receptors, the density of receptors in the plasma membrane of the target cells and numerous reactions within the target cells. The cellular portion of this system, in our case the FDC-P1 lactogenic assay, is a constant. Thus, only the ligand affinities for receptors at sites 1 and 2 are variables in this process. Mutations of the ligand that effect the agonist phase of the dose-response curve must influence binding at either site 1, site 2 or both sites. In contrast, mutations of the ligand that only effect the antagonist phase of the dose-response curve

Table 1
Agonist and antagonist activities and affinities of hGHs

Hormone	ED_{50}	ID_{50}	K_d
Wild-type hGH	12.1 nM	$\approx$5750 nM	0.047 nM
G120R hGH	19.5 nM	$\approx$3000 nM	0.047 nM
Y164E hGH	338.8 nM	$\approx$10000 nM	0.070 nM

must only effect site 1, because in receptor dimerization models, antagonist activities are determined only by the ligand and receptor concentrations and their affinity at site 1. Mutations of hGH within the functional epitopes of sites 1 or 2 demonstrate the validity of this reasoning. When functional epitopes within site 1 of hGH are mutated, both phases of the dose response curve are shifted in parallel, similarly, when functional epitopes within site 2 are mutated, the maximal response is limited [10,11].

Therefore, mutations external of the functional epitopes of sites 1 or 2, which shift the agonist phase of the dose response curve but do not affect the ID_{50} of the antagonist phase, must influence the function of site 2. Y164E hGH fills these criteria (Fig. 4 and Table 1) with a 29-fold reduction in the ED_{50} of the agonist phase and no measurable change (less than 2-fold) in the ID_{50} of the antagonist phase when compared to wild-type hGH. Thus, the Y164E mutation produces these effects by impairing the function of site 2.

Tyr^{164} is located on the opposite end of hGH from site 2. Tyr^{164} is a structural, but not a functional, epitope of site 1 [3,5]. Our mutagenic data support the conclusion that Tyr^{164} is not a site 1 functional epitope because this mutation affects neither the affinity for the hPRL receptor (Table 1) nor the antagonist phase of the dose-response curve (Fig. 4). These characteristics would be affected if Tyr^{164} was within a functional epitope of site 1. Further, this mutation has little or no influence on somatotropic activities of hGH (Table 1) or structural characteristics determined by spectroscopy (Fig. 2 and 3). Thus, the Y164E mutation is benign to the overall structure of hGH.

We interpret our data to suggest that Tyr^{164} may be part of a motif that undergoes the change of conformation induced by hGH binding to a lactogenic receptor at site 1 and documented by crystallographic structures (PDB number 1HGU) [5]. We suggest that disruption of this motif by mutagenesis reduces the effective transmission of the conformation change to site 2. Further work is in progress to identify the remaining residues that constitute this motif.

Fuh et al. [15] have previously used the FDC-P1 hPRL receptor assay to demonstrate the effects of the G120R mutation on hGH. The same general results were observed with G120R hGH having a severely reduced maximal activity. Several differences were also apparent. First, the activity of G120R hGH was reduced by approximately 80% in our work, while Fuh and colleagues showed a more substantial reduction in activity. Second, Fuh and colleagues' ED_{50}s were approximately an order of magnitude smaller and ID_{50}s were approximately an order of magnitude larger than our values. Although both studies were performed in FDC-P1 cells that were transfected with the 591 residue hPRL receptor, the number of receptors per cell was probably greater in the work by Fuh et al. Such a difference in receptor number would explain the differences in both agonist and antagonist phases of the dose-response curves for wild-type hGH. When either Fuh and colleagues [15] performed these same studies in Nb2 cells [21], they observed similar trends but with lower ED_{50}s and higher ID_{50}s. These results are consistent with the high number of prolactin receptors found in Nb2 cells. These data are quite different than those of Dattani et al. [22] who performed Nb2 assays. Dattani and colleagues observed similar maximal activities with either wild-type or G120R hGH, but a higher ED_{50} and lower ID_{50} when the G120R hGH was compared to wild-type hGH. These shifts in the dose-response curves are not expected if the G120R mutation affects site 2 and were not observed in our studies in FDC-P1 cells.

Mutations resulting in structural changes of proteins may affect functional properties and will provide clues to structure/function relationships of the protein. Crystallographic structures of hGH are available and useful when interpreting the effects of structural modifications generated by mutagenesis. The presence of a tyrosine at position 164 appears to promote the prolactin receptor binding at site 2 which may operate through a receptor binding-induced conformation change. Replacement of this hydrophobic residue with hydrophilic glutamic acid disrupts the arrangement of a hydrophobic pocket whose formation is promoted by binding of the prolactin receptor at site 1. Formation of this hydrophobic pocket is associated with rearrangement of local residues which are elements for the conformation change associated with restructuring the residues of hGH that comprise site 2. We have previously published mutagenic studies of Phe^{44} in hGH [12]. Phe^{44} is required for the lactogenic activities of hGH and articulates with Tyr^{164} upon ligand binding to the prolactin receptor to form a hydrophobic cluster. These residues may form a motif that regulate the site 2 function via site 1 binding.

In summary, we have identified by site-directed mutagenesis a residue that, despite being close to site 1 and distal to site 2, influences the ability of site 2 to function. Further, we have characterized the functional effects in lactogenic assays obtained by mutation of a hydrophilic residue within the structure of hGH. We believe this pattern of altered function, characterized by a right-shifted agonist action and unchanged antagonist actions, will be observed when elements of motifs transmitting binding-induced conformation changes are altered by mutation.

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