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The species specificity of growth hormone requires the cooperative interaction of two motifs

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Abstract Primate growth hormones (GH) activate both primate and non-primate somatotrophic receptors (GH receptors), but non-primate GHs do not activate primate GH receptors. Previous studies argued the interaction of Asp^{171} of human GH and Arg^{43} of the receptor produced an attractive ionic interaction. In non-primate GHs, His^{170} replaces the homologous Asp^{171}, producing a repulsive interaction with Arg^{43} of the primate receptor which was believed to reduce the attraction of non-primate GH for the human GH receptor, thus providing species specificity. In this report, H170D bovine GH had activity and affinity for human GH receptors approaching those of human GH. In contrast, replacing Asp^{171} of human GH with His did not significantly reduce somatotrophic activity, indicating that species specificity is not wholly explained by this residue's interaction with Arg^{43} of the receptor. Deletion of either Phe^{44} (a residue present only in primate GHs) or residues 32–46 (20-kDa form of human GH) each only marginally reduced somatotrophic activities. But the combination of the D171H mutation with either ΔPhe^{44} or Δ32–46 in human GH reduced binding and activity in a greater than additive fashion, indicated a functional interaction between these distant structural features. In bovine GH addition of phenylalanine at position 44 increased the somatotrophic activity and receptor affinity in cells containing the human GH receptor. The combination of the H170D mutation and the addition of phenylalanine at position 44 created a bovine GH with activity indistinguishable from wild-type human GH. Based on evidence from both bovine and human GHs, the cooperative interaction of these two distant motifs determined the species specificity and indicated that structural plasticity was a critical feature necessary for the species specificity of somatotrophic activity.

Key words: Growth hormone; Human; Bovine species; Somatotrophic receptor

1. Introduction

Primate growth hormones (GH) bind and activate both primate and non-primate GH receptors by dimerizing two receptors which bind at sites 1 and 2 on the ligand surface [1–5]; non-primate GHs bind and activate non-primate GH receptors but have very limited activity for primate GH receptors [6,7]. The ligand selectivity of the primate GH receptors was first recognized in 1956 [8] but has only recently been explored at the structural level using mutagenic studies with recombinant GHs and receptors. A comparative study of the human and bovine GH receptors by Laird et al. [9] first identified by mutagenesis Arg^{43} of the human GH receptor as a determinant of species specificity. Subsequent three-dimensional structures of trimeric complexes of human GH with the extracellular domain of the human GH receptor (GH binding protein, GHbp) (Protein Data Bank #3HHR) [10] showed Arg^{43} of the receptor formed two hydrogen bonds with Asp^{171} and Thr^{175} of human GH. Using this structural information and sequence alignment studies from several species Souza et al. [11] suggested that the ligand specificity displayed by primate GH receptors was among the 14 amino acids participating in the molecular interactions within the site 1 interface. Arg^{43} of the human GH receptor was replaced by leucine in all non-primate receptors while Asp^{171} of human GH was substituted by histidine in all non-primate GHs. All other residues participating in ligand–receptor interactions within site 1 were highly conserved. The increased size and charge of the histidine replacement at position 170[1] of non-primate GHs was suggested to be incompatible with Arg^{43} of primate GH receptors but compatible with Leu^{43} of non-primate GH receptors. In contrast to Asp^{171}, Thr^{175} of the GHs was conserved in all species examined. The role played by Asp^{171} and Arg^{43} has been investigated in several studies using site-directed mutagenesis [9,11–13] and most studies conclude that this interaction is critical for the observed species specificity.

These studies used a single static structural model (PDB #3HHR) to interpret their results. Recently Atwell et al. [14] showed that mutations can cause large changes in the ligand structure but that further structural changes created by mutagenesis can restore function despite the persistence of widespread structural changes. These data illustrate the plastic nature of human GH–receptor interaction. These studies illustrated the functional relationship of distant structural features with those within the energetically important epitopes [15] of the site 1 binding interface. The structural plasticity of this interface was demonstrated when Trp^{104} of the human GH receptor, a critical binding residue, was replaced by Ala. The plasticity of this interface was demonstrated by the altered spatial relationships between both residues at the bind-

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Abbreviations: GH, growth hormone; GH receptor, somatotrophic receptor; GHbp, GH binding protein; NHPP, National Hormone and Pituitary Program

[1] Residue 170 of bovine GH is analogous to residue 171 of human GH.

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ing interface and additional motifs within the ligand. This mutation (W104A-human GHbp) created a large cavity in the site 1 interface and decreased the affinity for wild-type human GH ($K_d > 1000$ nM). Mutation of human GH residues which surround Trp^{104} of the receptor were prepared by a phage display technique and screened for binding. This approach identified a penta-mutant of human GH (K168R, D171T, K172Y, E174A and F176Y) with a restored affinity ($K_d = 14$ nM). The three-dimensional structure of the W104A-human GHbp in a 1:1 complex with the penta-mutant human GH was resolved by X-ray crystallography (PDB #1AXI). This structure revealed local structural changes within energetically crucial epitope of the site 1 binding interface and substantial changes extending over the entire site 1 contact interface. Movements of up to 3 Å were detected for intermolecular contact residues that were up to 15 Å from the epicenter of this cluster of mutations. For example, a 3 Å salt bridge between Lys^{41} of human GH and Glu^{127} of the human GH receptor, located 11 Å from the mutational epicenter, was separated by an additional 2.4 Å in the mutant complex. This cavity was occupied by two ordered water molecules. These data suggested that residues 170 in non-primate and 171 in primate GHs, in addition to interacting with either Arg^{43} or Leu^{43} of primate or non-primate GH receptors, respectively, may also communicate with distant structural features that influence binding and activation of GH receptors.

The relationship of structural features which lie outside the functional determinants of site 1 to the structural plasticity and functional activities of the ligand has not been examined. To examine the importance of His in the Arg^{43}/Glu^{171} motif, the D171H human GH and a H170D bovine GH mutants were constructed. A static model of human GH would predict that replacement of Asp^{171} by His^{171} should mimic non-primate GHs and not bind the human GH receptor with high affinity, while replacement of His^{170} by Asp^{170} in bovine GH should allow for high affinity binding to the human GH receptor. Failure of this model would suggest that the structural plasticity of GHs may be affected by distant motifs which may influence species specificity.

Sequence alignment of GHs from various species showed that F44 is only present in primate GHs and thus may offer an additional determinant of species specificity. Deletion of Phe^{44} from human GH severely reduced its lactogenic activity, but produced little effect on somatotrophic activity [16]. Phe^{44} rests within the amino acids missing (residues 32–46) in the 20-kDa variant of human GH [17,18]. 20-kDa human GH retains a high affinity for the somatotrophic receptor. The biological activity of 20-kDa human GH has not been reported. Phe^{44} and the 15 amino acids deleted in the 20-kDa human GH are distant from Asp^{171}. The smallest distance between amino acids 32–46 and Asp^{171} of human GH is 11 Å; yet, Atwell's penta-mutation within the site 1 binding interface induces structural changes within amino acids 32–46 [14]. To investigate the possibility of an interaction between these distant motifs in human GH, double mutants containing the D171H mutation and the deletion of either amino acids 32–46 (Δ32–46/D171H) or Phe^{44} (ΔPhe^{44}/D171H) were constructed. Finally, bovine GH with Phe inserted between Ser^{43} and Ile^{44} (X44F) was constructed with or without the H170D mutation to determine the contributions of these two motifs in binding and activation of the human somatotrophic receptor within the structural context of the bovine protein.

2. Materials and methods

2.1. Plasmids and bacterial strains

An f_1 origin of replication was inserted at a unique *Cla*I site in pT7-7 plasmid (kindly provided by S. Tabor, Harvard Medial School, Boston, MA). The negative strand pT7-7 phagemid was used for cloning, production of single stranded (ss) DNA and expression of human GH. *Escherichia coli* strains DH5α, RZ1032 (dut^- and ung^-) and BL21(DE3) were used for cloning, uridine substituted ssDNA production and protein expression, respectively. A bovine GH expression system (#53024) was purchased from the American Type Culture Collection (ATCC). W3110 strain *E. coli* (ATCC #39936) was purchased for the expression of recombinant bovine GHs.

2.2. Recombinant phagemids and plasmids

The pT7-7 f(−) phagemid for the expression of wild-type human GH was previously described [16,19]. The pMON3209 bovine GH expression plasmid was purified from the purchased expression system. Restriction site information for the pMON3209 plasmid was kindly provided by Dr. G. Bogosian (Monsanto Corporation, Chesterfield, MO).

2.3. Site-directed mutagenesis

In vitro mutagenesis was performed by the Kunkel method [16,20]. Selection for positive mutants was facilitated by manipulation of restriction endonuclease sites through a primer-introduced translationally silent mutation. Complete DNA sequences in positive clones confirmed the presence of the desired mutation(s).

To construct mutants of bovine GH, the sequence encoding mature bovine GH was excised from pMON3209 using *Eco*RI and *Bam*HI and ligated into a similarly digested pT7-7f(−) phagemid. Mutagenesis was then performed as described above. Coding sequences containing the desired bovine GH mutations were inserted into the pMON3209 expression plasmid using these same restriction sites and sequenced to confirm the desired mutations.

2.4. Expression, folding and purification of human and bovine GHs

Purified phagemids containing wild-type, ΔPhe^{44}, 20-kDa, D171H, ΔPhe^{44}/D171H or 20-kDa/D171H human GH DNAs were transformed into BL21(DE3) cells and expressed as previously described [16,19].

Purified pMON3209 plasmids containing wild-type, H171D, X44F or H171D/X44F bovine GH were transformed into W3110 *E. coli*. One liter cultures of Vogel–Bonner medium [21] containing 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline were grown to an OD_{600} of 0.2–0.3 and protein expression induced for 4–6 h with 25 μg/ml 3β-indoleacrylic acid.

Human and bovine GHs were extracted, refolded and purified as previously described for human GH [16,19]. In short, *E. coli* were ruptured by a French pressure cell and the inclusion bodies collected by centrifugation. The inclusion bodies were dissolved in 4.5 M urea buffered by 100 mM Tris (not pHed). Following dissolution of the inclusion bodies and removal of bacterial debris, the pH was raised to between 11.0 and 11.5 to allow the rearrangement of disulfide bonds. The preparation was placed into dialysis against 20 mM Tris pH 9.0 to reduce the urea and bring the pH to that desired for purification, folding occurred during this process. GHs were purified by DEAE-cellulose (DE-52 Whatman, Clifton, NJ), dialyzed against 10 mM NH_4HCO_3, and lyophilized.

2.5. Characterization of recombinant proteins

Proteins were evaluated for size and purity by 12% SDS–PAGE under non-reducing and reducing condition [22]. Absorption and fluorescence spectra were collected to evaluate the folding of each protein at 20°C in 10 mM Tris pH 8.2, 150 mM NaCl.

2.6. FDC-P1 somatotrophic assay

FDC-P1 cells containing the human GH receptor were a gift from Genentech Inc. (South San Francisco, CA). Cells were maintained in RPMI 1640 containing 10 μM 2-mercaptoethanol, 1 nM human GH and 10% fetal calf serum [23]. Washed log phase cells were suspended in medium devoid of human GH and phenol red and returned to the incubator for 24 h prior to the assay. Stock concentrations of hormones were determined by the bicinchoninic acid/copper sulfate assay [24]. Hormones were diluted with phenol red-free medium to the de-

sired concentrations and added to triplicate wells of 96-well plates. Each well contained 15 000 FDC-P1 cells in a total volume of 100 μl. Plates were incubated at 37°C in a 5% CO_2/95% air atmosphere for 48 h. Proliferation was assessed by a vital dye method (Alamar blue, Accumed International, West Lake, OH) [16]. The data were used to calculate an ED_{50} by a four-parameter fit method [25].

2.7. Receptor binding assays

Receptor binding assays were performed with temperatures that were less than the melting temperatures of plasma membranes; therefore receptor binding data primarily reflected binding at site 1 of the GH ligands. Human GH was iodinated with iodogen (Pierce Chemical Co., Rockford, IL) and carrier-free [^{125}I]iodine to a specific activity of 66 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 $ZnSO_4$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, approximately 1 ng of [^{125}I]human GH and various concentrations of human or bovine GHs. Incubations were run for 20 h at room temperature. Membranes were collected by centrifugation and membrane-associated [^{125}I]human GH measured. Relative affinities were calculated by the method of Scatchard [26].

3. Results

3.1. Characterization of recombinant proteins

DNA sequencing confirmed the presence of wild-type human [27] and bovine GHs [28] and the desired mutations. Final yields for human and bovine GHs were typically between 10 and 40 mg/l of fermentation. Recombinant human or bovine GHs co-migrated with National Hormone and Pituitary Program (NHPP) human or bovine GHs, respectively, on a 15% SDS polyacrylamide gel under reducing conditions; co-migration of mutants containing the Δ32–46 mutation with the 20.1-kDa molecular weight marker is consistent with the calculated molecular weight of 20.3 kDa (Fig. 1B). All recombinant proteins were homogeneous. Co-migration of recombinant proteins with the appropriate NHPP standards on a non-reducing 15% SDS polyacrylamide gel suggested that all proteins folded to produce correct disulfide bonds (Fig. 1A) [19]. Human GHs containing the Δ32–46 mutation migrated faster than the NHPP human GH under non-reducing conditions and appeared to be folded as a single product. Several human GHs formed small amounts of dimeric hormone that were evident on non-reducing gels (Fig. 1A). Dimeric hormones were not evident for the bovine GH preparations under non-reducing conditions.

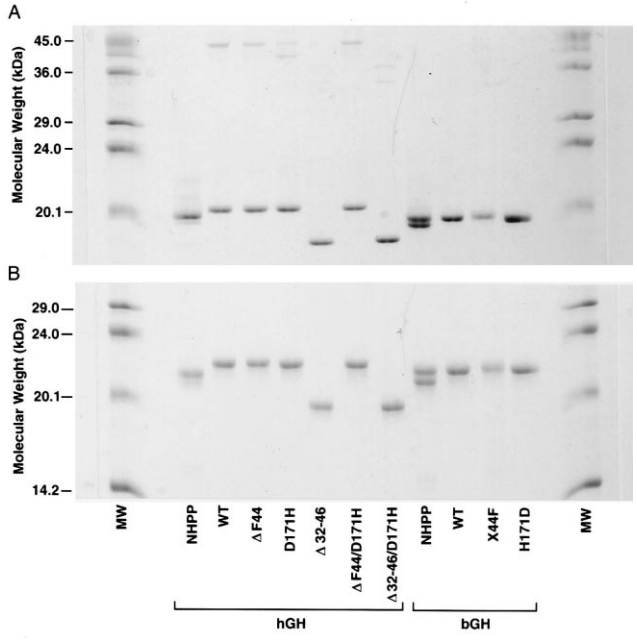

Fig. 1. Non-reducing (A) and reducing (B) SDS gel electrophoresis. Electrophoresis of NHPP and recombinant human and bovine GHs was performed on 12% polyacrylamide gels according to the method of Laemmli [22]. Proteins were in a solution containing 60 mM Tris–HCl, 10% glycerol, 1% SDS, 0.01% pyronin-Y with or without 1% 2-mercaptoethanol. Each lane contains 5 μg of protein.

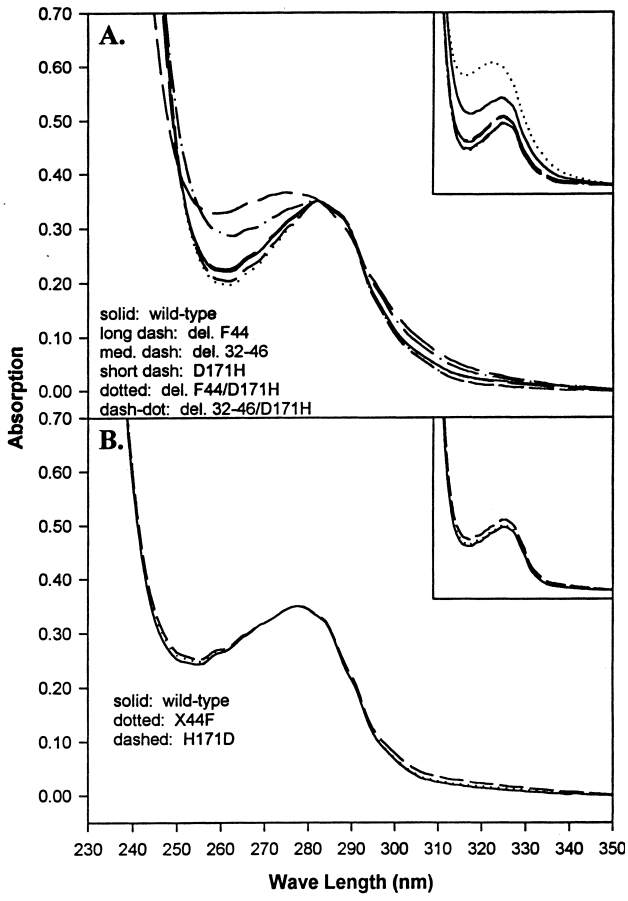

Fig. 2. UV absorption spectra for human (A) and bovine (B) GHs. Protein concentrations were 20 μM for GHs. All proteins were in a solution containing 10 mM Tris–HCl, pH 8.2, containing 150 mM NaCl. Spectra presented are the average of three scans and were obtained at ambient temperature on a Uvicon model 930 spectrophotometer. The raw data (insets) were normalized to absorption at 277 nm.

3.2. Spectroscopy of the recombinant proteins

Normalized absorption spectra (Fig. 2A) of the wild-type and ΔPhe^{44} human GHs overlie each other. Deletion of amino acids 32–46 in human GH produced a blue shift in the maximum absorption from 277 to 269 nm and increased the overall absorption. All human GHs containing the D171H mutation show a decrease in absorption in the 270–290 maxima (Fig. 2A). Addition of the D171H mutation to Δ32–46 human GH ameliorates the blue shift and returns the maxima to 277 nm as in wild-type human GH. When the spectra are normalized for absorption at 277 nm, D171H and ΔPhe^{44}/D171H have decreased absorptions in the 245–270 nm region while

the absorptions of Δ32–46 and Δ32–46/D171H are increased relative to the absorption of wild-type human GH.

Normalized absorption spectra (Fig. 2B) of wild-type, X44F and H171D bovine GHs overlie each other. All recombinant bovine GHs have absorption maxima at 277 nm.

The shapes and maxima of the fluorescence spectra (excitation 285 nm) (Fig. 3A) are similar for wild-type, ΔPhe^{44}, D171H and ΔPhe^{44}/D171H human GHs. The spectral maxima of the Δ32–46 and Δ32–46/D171H human GHs were red-shifted by 2 nm but maintained a shape similar to wild-type human GH. Δ32–46 human GH appeared to have a reduced quantum yield.

The shapes of the fluorescence spectra (excitation 285 nm) (Fig. 3B) are similar for all recombinant bovine GHs. Replacement of His^{170} with Asp blue-shifted the maxima 5 nm from 336 nm to 331 nm and reduced the quantum yield relative to wild-type bovine GH (see inset in Fig. 3B). The maximum of X44F bovine GH was blue-shifted 2 nm when compared with the wild-type bovine GH.

3.3. Somatotrophic activities of recombinant proteins

Activities of human and bovine GHs were assessed in the

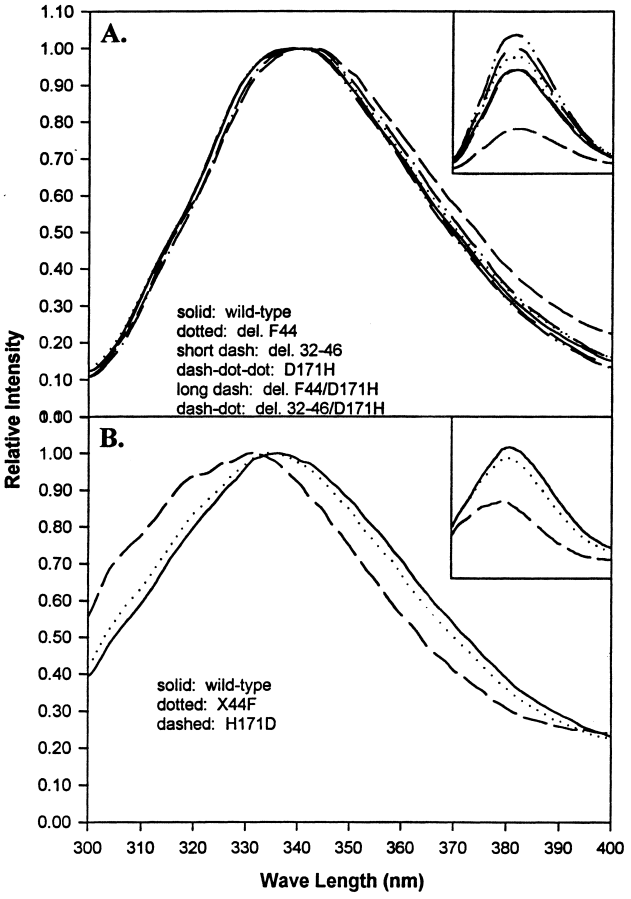

Fig. 3. Fluorescence spectra for human (A) and bovine (B) GHs. Protein concentrations were 3.3 and 6.7 μM for human and bovine GHs, respectively. Proteins were in a solution containing 10 mM Tris–HCl, pH 8.2, containing 150 mM NaCl. Spectra presented are the average of three scans. The emission spectra were collected with a Perkin-Elmer LS-50B at ambient temperature with a 285 nm excitation. The raw data (insets) for each protein were normalized where the maximum relative fluorescence was set equal to 1.0.

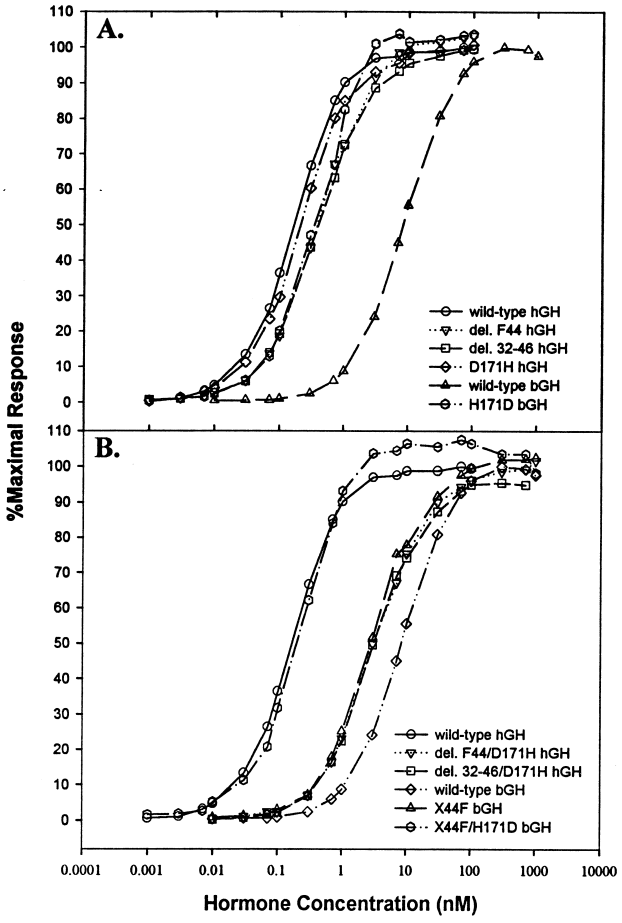

Fig. 4. Somatotrophic activity of a series of single (A) and double (B) mutants of human and bovine GH. Recombinant GHs were suspended in 50 mM ammonium bicarbonate. Protein concentrations were determined and dilutions in media were added to FDC-P1 bioassays as described in Section 2. Data are representative of three separate experiments. The maximum responses of each hormone were normalized to the maximal response of their respective wild-type GH.

FDC-P1 somatotrophic bioassay (Fig. 4, Table 1). Replacement of Asp^{171} with histidine in human GH provided an ED_{50} of 206 pM which was indistinguishable from wild-type human GH (ED_{50} = 162 pM) (Fig. 4A). Elimination of Phe^{44} (ΔPhe^{44}) or amino acids 32–46 (Δ32–46) in human GH increased the ED_{50}s to 413 and 392 pM, respectively (Fig. 4A). This small increase in the ED_{50} for ΔPhe^{44} is consistent with our previously results [16]. The ED_{50}s of the doubly mutated (ΔPhe^{44}/D171H and Δ32–46/D171H) human GHs were increased approximately 20-fold to 3265 and 2956 pM, respectively, when compared to wild-type human GH (Fig. 4B, Table 1). These increased ED_{50}s for the double mutants were unexpected since each single mutant only minimally affected somatotrophic activity; ΔPhe^{44} human GH was the least potent single mutant with an ED_{50} increased by 2.5-fold relative to wild-type human GH. Each of the double-mutated human GHs had somatotrophic activities only marginally greater than wild-type bovine GH (2–3-fold). A structural interaction between these two positions is indicated by the change in activity of the double mutants being larger than the sum of the effects of the individual mutants [21].

Replacement of His^{170} of bovine GH with Asp enhanced

the somatotrophic activity via the human GH receptor reducing the ED_{50} 22-fold from 8329 pM to 369 pM (Fig. 4A). The somatotrophic activity of H171D bovine GH was only 2.3-fold less than that for wild-type human GH. In our hands the FDC-P1 somatotrophic assay can reliably distinguish a two-fold change in ED_{50}. Insertion of Phe between Ser^{43} and Ile^{44} of the wild-type bovine GH decreased the ED_{50} 2.8-fold to 2947 pM. The ED_{50} for X44F bovine GH is indistinguishable from Δ32–46/D171H human GH. The X44F/H170D bovine GH double mutant had an ED_{50} improved by 37-fold when compared with wild-type bovine GH to 227 pM. The ED_{50} of X44F/H170D bovine GH is indistinguishable from wild-type human GH.

The double-mutated bovine GH was a potent mimic of wild-type human GH. When the ED_{50} of X44F/H170D bovine GH was compared to the ED_{50}s of either the single-mutated or wild-type bovine GHs (Table 1, ratios in parentheses), the sum of the effects of single mutations (X44F: 13-fold and H170D: 1.6-fold) in reducing activity was less than the reduction observed with wild-type bovine GH (36.6-fold). The greater than additive effects of these two motifs in bovine GH document their interaction when activating the human GH receptor.

3.4. Receptor binding of the recombinant proteins

Correlation coefficients for all human GHs by Scatchard analyses were >0.82 (Table 1). Wild-type human GH had a K_d of 0.30 nM which was in close agreement with published values [29]. Replacement of Asp^{171} with histidine increased the K_d five-fold to 1.6 nM. Elimination of Phe^{44} or amino acids 32–46 increased the K_ds approximately 10-fold to 3.5 and 2.4 nM, respectively. The human GHs with mutations in both motifs (ΔPhe^{44}/D171H and Δ32–46/D171H human GH) lessened the binding affinities by greater than 100-fold producing K_ds of 41 and 42 nM, respectively. These values were increased approximately 10–30-fold over the binding affinities of any single human GH mutation. The K_ds and ED_{50}s for wild-type and mutant human GHs were highly correlated ($r^2 = 0.99$). Again, this second measure of activity failed to follow the additive rule [21] and suggested a structural interaction between these two motifs of human GH.

Correlation coefficients for the Scatchard analyses of bovine GHs were >0.78. The K_ds of wild-type and X44F bovine GH for the human GH receptor were 39 and 18 nM, respectively (Table 1). These K_ds are approximately 100-fold higher than that of wild-type human GH. In contrast, the K_d of H170D bovine GH was decreased 28-fold ($K_d = 1.4$ nM) to a value less than five-fold greater than that for wild-type human GH. The K_ds and ED_{50}s for the bovine GHs were highly correlated ($r^2 = 0.98$).

4. Discussion

If the species specificity of the association between GHs and the human GH receptor is principally determined by the ionic interaction between Arg^{43} of the receptor and the ionic character of the ligand's residue at position 170 or 171, then placing an acidic residue at position 170 of bovine prolactin should provide full activity and placing a positively charged residue at position 171 of human GH should greatly reduce the activity. We observed that replacement of His^{170} with aspartic acid in bovine GH decreased the ED_{50} (369 pM) in the FDC-P1 somatotrophic assay by 23-fold when compared with wild-type bovine GH (8329 pM) (Table 1). This decrease in ED_{50} was paralleled by a 28-fold increase in affinity for the human GH receptor (Table 1). The ED_{50} and K_d of H170D bovine GH were 2.3- and 4.7-fold less, respectively, than the values for wild-type human GH. These results confirmed the studies of Laird et al. [9], Souza et al. [11] and Behncken et al. [13] and were consistent with the hypothesis that the specific interactions between Arg^{43} of the human GH receptor and amino acid 170 of the non-primate GH ligands contributed to the species specificity of the primate GH receptors.

In contrast, replacement of Asp^{171} with His in human GH (D171H) failed to significantly increase the ED_{50} (only 1.3-fold) while increasing the K_d by 5.0-fold (Table 1). These results were consistent to the observations of Behncken et al. [13] and opposite to what would be expected if the simple ionic model completely explained species specificity. Gobius et al. [12] reported that *rabbit* GH receptors which have been humanized by the replacement of the Leu^{43} with arginine did not distinguish between primate and non-primate GHs. Our results and those of Gobius et al. [12] indicate that additional structural features play a role in the species specificity of the human GH receptor.

The structural changes created by mutation of GHs pro-

Table 1
ED_{50} and K_d values for human and bovine growth hormones

Hormone	ED_{50} value for biological activity		Relative binding affinity	
	ED_{50} (pM)	Ratio[b]	K_d (nM)	Ratio
WT hGH	162 ± 4.7[a]	1.0	0.30	1.0
ΔPhe^{44} hGH	413 ± 12.5	2.5	3.5	12.0
Δ32–46 hGH	392 ± 12.5	2.4	2.4	8.0
D171H hGH	206 ± 6.3	1.3	1.6	5.0
ΔPhe^{44}/D171H hGH	3265 ± 106.8	20.1	41.0	135
Δ32–46/D171H hGH	2956 ± 98.3	18.2	42.0	140
WT bGH	8329 ± 241.5	51.3 (36.6)	39.0	130
X44F bGH	2947 ± 91.8	18.2 (13.0)	18.0	60
H170D bGH	369 ± 10.0	2.3 (1.6)	1.4	4.7
X44F/H170D bGH	227 ± 6.2	1.4 (1.0)	–	–

Average coefficient of variation for ED_{50} determinations = 3.0%
Correlation between ED_{50} and K_d for human GHs: $r^2 = 0.99$
Correlation between ED_{50} and K_d for bovine GHs: $r^2 = 0.98$
[a]Mean ± S.D.
[b]Ratios without parentheses are relative to WT hGH, ratios in parentheses are relative to X44F/H170D bGH.

duced parallel changes in somatotrophic activity and binding to the human GH receptor ($r^2 = 0.98$–0.99). Binding studies using membrane fragments were performed at room temperature which is typically below the melting point of membrane lipids. This condition would not allow the lateral diffusion of membrane-bound receptors, thus, primarily allowing binding to be measured at site 1 of the GH. In addition, both residue 171 and the residues comprising minihelix 1 were structural determinants of site 1 [10,15]. These data indicated that the species specificity of these GHs was regulated by site 1 binding.

Deletion of Phe^{44} or amino acids 32–46 of human GH increased the ED_{50}s only 2–3-fold (Table 1), a change of similar size to that produced in D171H human GH. In contrast, the ED_{50}s of doubly mutated human GHs (ΔPhe^{44}/D171H and Δ32–46/D171H) were increased approximately 20-fold. This reduction of activity was greater than the sum obtained from individual mutations [30]. These data indicated that these distant motifs have a functional interaction. Amino acid 171 and amino acids 32–46 are at least 11 Å apart. This distance does not allow a direct physical interaction of these two motifs. Thus, the interaction between these sites must be mediated by additional intervening residues.

Our results with bovine GH parallel those with human GH. Bovine GH mutated in these two motifs (X44F/H170D) mimicked the activity of human GH (Table 1). Deletion of Phe^{44} or the presence of a histidine in position 170 reduced the biological activity 13- or 1.6-fold, respectively. Wild-type bovine GH was 36-fold less active than the doubly mutated protein. As with human GH, the sum of the individual functional changes was much less than the reduction of activity of wild-type bovine GH compared to doubly mutated bovine GH. Despite the two proteins having only 67% homology, this relationship described a functional interaction of these motifs in bovine GH when activating the human GH receptor.

The absorption spectra (Fig. 2A) collected for the human GHs argued for structural perturbations associated with either the D171H or Δ32–46 mutations. In contrast, no structural perturbations were detected when Phe^{44} was eliminated from human GH. Replacement of Asp^{171} by His decreased absorption in the 245–270 nm region of the absorption spectra (Fig. 2A) for all human GHs containing this mutation. In contrast, large increases in absorption were observed across the entire absorption spectrum for Δ32–46 deletion mutants, possibly resulting from the hydration of tyrosines 160 and 164 and rearrangement of the disulfide bond angle and/or distance between cysteines 53 and 165 (Fig. 2A). In doubly mutated human GH, one mutation appeared to offset the absorption effects of the other mutation. A large perturbation of the four-helix bundle of the human GHs is also argued against by the small changes observed in the fluorescence spectrum (Fig. 3A) which largely represent the status of Trp^{86} contained within the core of the four-helix bundle.

Mutational studies of enzymes have demonstrated additive effects when single mutants were combined in a protein [30]. However, the rule of addition breaks down under two conditions. The first condition occurs when the mutated residues interact with each other and no longer behave independently. These interactions can be the result of physical contact or indirect contact through electrostatic interactions or structural perturbations. The second condition arises when the mutation(s) alters either the reaction mechanism or the rate-limiting step. The rule of addition also applies for multiple mutations in protein–protein interactions, protein–DNA interactions or protein stability.

When compared to wild-type human GH, wild-type bovine GH had naturally occurring differences in both motifs, i.e. the absence of Phe at position 44 and the presence of His at position 170. Thus, ΔPhe^{44}/D171H human GH would be predicted to be the best mimic of bovine GH. This doubly mutated human GH has only a three-fold greater activity than wild-type bovine GH and a 20-fold lower activity than wild-type human GH. Thus, these two structural differences can generally account for the differences in activities of human and bovine GHs.

In this and other work, predictions based on a static structural model were only partially effective at predicting the results of mutations. The recent evidence of GH plasticity in receptor recognition [14] demonstrates that mutations at the binding interface have far-reaching structural effects and that mutations distant from the binding interface can have dramatic effects on the function of a protein [31]. In this work we have demonstrated that distant mutations have cooperative effects with mutations within the functional somatotrophic binding interface.

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