Aspartate 171 Is the Major Primate-specific Determinant of Human Growth Hormone

ENGINEERING FORCINE GROWTH HORMONE TO ACTIVATE THE HUMAN RECEPTOR*

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It has been known for more than 4 decades that only primate growth hormones are effective in primate species, but it is only with the availability of the 2.8 Å structure of the human growth hormone (hGH)-hGH-binding protein (hGHBP)2 complex that Souza and co-workers (Souza, S. C., Frick, G. P., Wang, X., Kopchick, J. J., Lobo, R. B., and Goodman, H. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 959–963) were able to provide evidence that Arg-43 on the primate receptor is responsible. Here we have examined systematically the interaction between Arg-43 (primate receptor) or Leu-43 (non-primate receptors) and their complementary hormone residues Asp-171 (primate GH) and His-170 (non-primate hormones) in a four-way comparison involving exchanges of histidine and aspartate and exchanges of arginine and leucine. BAF/B03 lines were created and characterized which stably expressed hGH receptor, R43L hGH receptor, rabbit GH receptor, and L43R rabbit GH receptor. These were examined for site affinity, for the ability to bind intact cells, and for proliferative biopotency using hGH, D171H hGH, porcine GH, or H170D porcine GH. We find that the single interaction between Arg-43 and His-170/171 is sufficient to explain virtually all of the primate species specificity, and this is congruent with the crystal structure. Accordingly, for the first time we have been able to engineer a non-primate hormone to bind to and activate the human GH receptor.

The growth hormone receptor (GHR)* is a member of the hematopoietic cytokine receptor family, sharing common structural and functional features with receptors for prolactin, erythropoietin, granulocyte and granulocyte-macrophage colony-stimulating factors, several interleukins, thrombopoietin, ciliary neurotrophic factor, oncostatin M, and leptin (for review, see Refs. 1 and 2). Of this family, the interaction of GH with its receptor is the best characterized, primarily because of the extensive structure/function studies that have been carried out on both GH and the GHR (for review, see Ref. 1) and because it is the only member for which the crystal structure of the hormone-receptor complex is known (3). Both crystal structure and solution studies support the concept that two identical hormone-binding subunits bind the helix bundle hormone through similar loop determinants on the receptor β-sandwich structures. The hormone is captured by receptor 1 through binding to determinants located in a 900-Å2 patch encompassing helices 1 and 4 and the unstructured loop between helices 1 and 2. Eight key residues account for 85% of the binding energy, with electrostatic interactions governing the approach of hormone to the receptor binding site (4, 5). Electrostatic interactions are also important specificity determinants because 5 of the 7 residues that were modified to enable prolactin to bind to the GH receptor with high affinity involved charged residues (6).

GHs from humans and monkeys (primate GHs) are unique in that they are able to bind with and activate non-primate GHRs as well as primate GHRs, whereas the GHs from non-primates are ineffective in primates (for review, see Ref. 7). To elucidate which residues on the receptor are responsible for this species specificity of binding, sequence alignment analysis was performed on human GHR (hGHR) and a number of non-primate GHRs in conjunction with an examination of the crystal structure of the GH-GHBP2 complex. Of residues within the five major loops involved in hormone binding (2), the interaction between Arg-43 of the human receptor and Asp-171 of the human hormone is striking because in non-primate receptors this position is replaced by leucine, and histidine occupies the place of aspartate in non-primate hormones (the equivalent position in porcine and bovine GH is 170). An unfavorable charge repulsion/steric hindrance between hormone His-170 and receptor Arg-43, rather than a favorable salt bridge between this arginine and primate hormone Asp-171, could be an important element in the inability of non-primate hormones to bind to the human receptor. Accordingly, we previously undertook site-directed mutagenesis at position 43, converting Leu-43 of the rabbit receptor to arginine (L43R rbGHR), and compared the ability of wild type and mutant receptors to discriminate between binding of hGH and bovine GH (8). We were disappointed to find that the L43R rbGHR mutant was able to bind human GH (hGH) and bovine GH (bGH) with almost identical affinity, indicating that it was not the species specificity determinant (8). However, a more recent study on bovine and rat GHRs by Souza et al. (9) has revealed that conversion of leucine to arginine at position 43 of these receptors severely abrogated the binding ability of bGH and also reduced its signaling ability. Based on the crystal structure of

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* The abbreviations used are: GHR, growth hormone receptor; GH, growth hormone; (GHBP)2, GH-binding protein; h, rb, b, and p prefixes indicate human, rabbit, bovine, and porcine, respectively; MAb, monoclonal antibody; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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the GH-(GHBP)_2 complex, Souza et al. (9) again proposed that an unfavorable interaction between Hia-170 on non-primate GH and Arg-43 on the human receptor accounts for primate species specificity of binding.

In this study, we have sought to resolve this conflict. We have examined the binding and signaling characteristics of rabbit and human GHRs with human, porcine, and bovine GHs as well as the effect of exchanging both the receptor leucine for arginine and the hormone histidine for aspartate. This has allowed us to bind a non-primate GH to the human receptor for the first time and to resolve the conflict between our previous study and that of Souza et al. (9).

EXPERIMENTAL PROCEDURES

Hormone Mutagenesis and Expression—Preparation of recombinant pGH and H170D pGH has been described previously (10, 11). Recombinant bGH was a gift from Cyramid (Princeton NJ). Human GH and the D171H hGH analog were expressed in Escherichia coli and purified to greater than 98% homogeneity (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the manner described in Ref. 10, except that the pH of the refolding buffer and Q-Sepharose loading buffer was 8.2. The D171H mutation was introduced into the coding sequence of the hGH cDNA using the Altered Sites mutagenesis procedure (Promega). In this procedure, an oligonucleotide encoding the desired mutation is annealed to single strand hGH-pSelect, and the complex is incubated with T4 DNA polymerase and T4 DNA ligase (Promega) to produce double stranded circular plasmid according to instructions from Promega. This was then subcloned into the pC611 vector (kindly provided by BresaGen, Adelaide, Australia) for expression, as described before (10). The mutation was confirmed by dideoxy sequencing (U.S. Biochemical Sequense kit) of the complete D171H coding region. Hormone concentrations were determined spectrophotometrically using an extinction coefficient of ε_290 = 18,200 M^-1 cm^-1.

Receptor Mutagenesis—Site-directed mutagenesis was undertaken on the rabbit GHR (rbGHR) and hGHR cDNA using the Altered Sites mutagenesis procedure as described previously (8). Mutant sequences were then subcloned into the pECE expression vector. Creation of rabbit L43R and human R43L GHR mutants and the absence of other sequence changes were verified by dideoxy sequencing.

Establishment of Cell Lines Expressing GHRs—The interleukin-3-dependent cell line BAF/B03 has been described previously (12). Cells were routinely passaged in 5% CO_2 at 37 °C in RPMI 1640 medium supplemented with 5 μg/ml gentamicin, 10% serum supernatant (a fetal bovine serum alternative supplied by Biowhittaker through Edward Keller Australia Ltd., Underwood Queensland) and 50 units/ml interleukin-3 (generous gift from Dr A. Hapel, John Curtin School of Medical Research, Australian Capital Territory, Australia). Cell confluency of 70% was reached within 15 days with an increase of 10^4 cells/ml growth medium as described in Ref. 11.

Characterization of Cell Lines Expressing GHRs—BAF/B03 cells were prefered over FDC-P1 cells in this study as we have found that they express GHRs at a higher level than FDC-P1 cells. Whole cell Scatchard analysis (at least three independent assays where 125I-hGH was displaced by unlabeled hGH for each cell line) revealed an affinity for hGH of 2.9 ± 0.2 × 10^9 M^-1 with 7,419 ± 777 receptors/cell for BAF/B03-hGH cells, an affinity of 3.3 ± 0.2 × 10^9 M^-1 with 3,164 ± 528 receptors/cell for BAF/B03-L43R hGHR cells, and an affinity of 5.0 ± 0.4 × 10^9 M^-1 with 2,852 ± 455 receptors/cell for BAF/B03-L43R rGHR cells. No specific 125I-
hGH binding was seen in nontransfected BAF/B03 cells. The fact that ED50 values for proliferative biopotency are around 0.05 \times 10^{-3} M (Tables I–IV, see below) suggests that there is a substantial number of spare receptors in these lines.

**Binding and Signaling with the Human Receptor**—Table I presents the combined data for wild type human GH receptor-expressing cells, and Fig. 1, A–C, presents representative data in graphic form. First, it can be seen that substitution of histidine for aspartate at position 171 of hGH has only a small effect (about a 2-fold reduction) on binding in the whole cell assay and on biopotency. However, in the solubilized assay where dimerization cannot occur, this substitution is seen to have a considerable impact on site 1 affinity (about a 40-fold decrease). Porcine GH is seen to bind very poorly in the whole cell assay (about 4,000-fold less than hGH) and to be very poorly effective in the bioassay (about 2,300-fold less). In the solubilized receptor assay, pGH effectively does not bind to the hGHR. However, substitution of aspartate for histidine at position 170 of pGH results in a hormone that is only 24-fold less than hGH in whole cell binding affinity and 14-fold less in biopotency. This is essentially a result of increased site 1 interaction because in the solubilized receptor assay the affinity of H170D pGH for the hGHR is only about 30-fold lower than hGH.

The converse study, where Arg-43 in the hGHR is replaced by a leucine, shows that D171H hGH now binds and signals as effectively as wild type hGH (Table II). Against the R43L hGHR, pGH now behaves similarly to the way H170D pGH does against the wild type human receptor, i.e. its whole cell affinity is about 15-fold less than hGH, its biopotency is about 14-fold less than hGH, and in the solubilized receptor assay its affinity is only 30-fold less than hGH. Thus, mutation of either receptor Arg-43 to non-primate leucine or mutation of hormone Asp-171 to non-primate histidine has a very similar effect on magnitude of binding affinity and biopotency. This effect is also seen with another non-primate GH, bGH, which binds and acts poorly against the wild type human receptor but is quite effective (3–4-fold less than hGH) against human receptor with leucine in position 43.

**Binding and Signaling with the Non-primate Receptor**—Table III presents the combined affinity and biopotency data for the same GH analogs, but in this case against the rbGHR. It can be seen that replacement of Asp-171 of hGH with histidine has little effect on binding or bioactivity. Again there is a small loss in solubilized receptor affinity, suggesting that aspartate is slightly more effective in this position against receptor Leu-43, but clearly either histidine or aspartate sits well against a leucine at receptor residue 43. Porcine GH is considerably less effective than hGH against the rabbit receptor in accord with our previous data (11). This would appear to be primarily a result of poor site 1 interactions because the affinity of pGH in the solubilized receptor assay is markedly less than hGH. This poor interaction is evidently not a result of hormone residue 170 interactions because substituting aspartate for histidine at this position again improves solubilized receptor affinity by only 2–3-fold, against leucine at position 43. Bovine GH is seen to bind and act considerably more effectively than pGH against the rabbit receptor (Table III).

Creation of a “humanized” rabbit receptor by replacing Arg-43 with leucine provides the final argument for the importance of this interaction in determining primate specificity (Table IV). As with the wild type rabbit receptor, hGH binds more strongly than it does against its own receptor. However, replacing Asp-171 with histidine markedly decreases (about 50-fold) site 1 affinity. Evidently strong site 2 interactions are able to compensate in that the whole cell affinity and biopotency are not markedly (about 2-fold) different from wild type hGH. Porcine GH now binds very poorly in the whole cell assay and effectively does not bind in the solubilized receptor assay. Biopotency is also very markedly reduced. This situation is
Binding affinities were determined by Scatchard analysis, and fold changes were calculated within each experiment. Proliferative ability was “Experimental Procedures”). Arrows indicate increase or decrease relative to wild type hGH (shown as unity). Whole cell and solubilized receptor determined.

Species Specificity of Growth Hormone Binding

**Table II**

For experimental details, see legend of Table I.

| Hormone | Affinity          | Whole cell     | Proliferative ability |
|---------|-------------------|----------------|-----------------------|
| hGH     | 1 (K_a = 6.32 ± 1.76 x 10^9 M^-1) | 1 (K_a = 3.32 ± 0.21 x 10^9 M^-1) | 1 (ED_50 = 4.54 ± 0.50 x 10^-11 M) |
| D171H hGH | 1.7 ± 0.3 ↓      | 1.1 ± 0.2 ↓    | 1.2 ± 0.3 ↑           |
| pGH     | 27.2 ± 7.9 ↓     | 14.5 ± 2.1 ↓   | 13.5 ± 3.0 ↓          |
| H170D pGH | 4.6 ± 1.1 ↓     | 20.8 ± 1.4 ↓   | 15.3 ± 3.9 ↓          |
| bGH     |                    | 3.5 ± 0.6 ↓    | 3.6 ± 1.5 ↓           |

* ND, not determined.

**Table III**

Fold changes of various hormones with wild type hGHR (relative to wild type hGH)

| Hormone | Affinity          | Whole cell     | Proliferative ability |
|---------|-------------------|----------------|-----------------------|
| hGH     | 1 (K_a = 5.38 ± 0.97 x 10^9 M^-1) | 1 (K_a = 5.95 ± 0.57 x 10^9 M^-1) | 1 (ED_50 = 5.73 ± 0.50 x 10^-11 M) |
| D171H hGH | 1.5 ± 0.2 ↓      | 1.2 ± 0.1 ↓    | 1.6 ± 0.3 ↓           |
| pGH     | 179.2 ± 72.0 ↓   | 8.3 ± 1.1 ↓    | 5.4 ± 0.4 ↓           |
| H170D pGH | 40.4 ± 9.9 ↓    | 20.6 ± 3.1 ↓   | 11.6 ± 0.7 ↓          |
| bGH     | 3.5 ± 0.9 ↓      | 2.0 ± 0.6 ↓    | 1.3 ± 0.2 ↓           |

**Table IV**

For experimental details, see legend of Table III.

| Hormone | Affinity          | Whole cell     | Proliferative ability |
|---------|-------------------|----------------|-----------------------|
| hGH     | 1 (K_a = 7.53 ± 0.78 x 10^9 M^-1) | 1 (K_a = 4.99 ± 0.41 x 10^9 M^-1) | 1 (ED_50 = 3.68 ± 0.68 x 10^-11 M) |
| D171H hGH | 52.3 ± 15.1 ↓    | 1.7 ± 0.2 ↓    | 1.9 ± 0.3 ↓           |
| pGH     | >10,000,000 ↓    | 125.5 ± 26.9 ↓ | 256.0 ± 36.3 ↓        |
| H170D pGH | 65.2 ± 19.5 ↓   | 20.6 ± 2.1 ↓   | 12.1 ± 2.7 ↓          |
| bGH     | 690.8 ± 72.4 ↓   | 2.6 ± 0.1 ↓    | 37.4 ± 8.6 ↓          |

effectively reversed by substitution of His-170 with aspartate, so that this analog now behaves similarly to pGH binding in its binding to the wild type rabbit receptor. In support of both our previous study (8) and the study of (9), bovine GH binding to the L43R rabbit receptor is only about 2.5-fold lower than binding to wild type rabbit receptor, yet in the solubilized receptor assay its site 1 binding is very poor (700-fold less), and its biopotency is reduced quite strongly (about 35-fold).

**Proliferative Bioassays—MTT assays were used as described previously (11) to compare the biopotencies of the various hormone preparations against wild type or mutant GHR-expressing cells. Because two GHRs interact with one GH molecule and because they use virtually the same residues to interact with the hormone at either site 1 or site 2 (3, 14), it is not possible to compare the relative biopotencies between wild type and mutant cell lines because of the uncertainty that the mutation could be affecting the bioactivity through site 2 interactions and not site 1 interactions. For this reason the biopotencies of the GHS with different cell lines are compared relative to wild type hGH.

**Bioassay with hGH Receptor-expressing Lines—**With the hGH-expressing cell line, the D171H hGH mutant was similar to wild type in its biopotency (see “Discussion”). The non-primate GHS, bGH and pGH, were very poor agonists for this cell line, showing 235- and 2,342-fold lower biopotency relative to hGH. For pGH, the introduction of the H170D mutation improved the biopotency such that it is only 14-fold lower than that of hGH, an improvement of greater than 150-fold. Similar improvements in biopotency were observed with bGH and pGH upon the introduction of the R43L mutation in the hGHR. In this case, the biopotency of these hormones relative to hGH became only 3.6- and 13.5-fold lower than hGH, an improvement of more than 80- and 150-fold, respectively, when compared with the wild type hGHR-expressing cell line. In contrast, the H170D pGH analog exhibited similar biopotencies against the wild type hGHR- and R43L hGHR-expressing cell lines, indicating that aspartate at this location is equally well tolerated against either arginine or leucine.

**Bioassay with rbGH-expressing Lines—**With the wild type rabbit receptor, hGH, D171H, and bGH have similar high potencies. pGH is a poor agonist, and H170D pGH is reduced further in potency by a factor of 2. Introduction of Arg-43 leads to a marked fall in biopotency for pGH and bGH but no change in biopotency for H170D pGH. Again, the D171H hGH analog appears able to compensate for loss of site 1 affinity against the L43R rbGH.

**Correlations between Bioactivity (ED_50) and Affinity—**Correlation analysis using all of the data in Tables I–IV (except for the hGH/L43R combination) is shown in Fig. 2. It can be seen that site 1 affinity does not correlate with biopotency, whereas whole cell affinity correlates very well (r = 0.99, p < 0.001). This indicates that the whole cell assay measures formation of the trimeric signaling complex.
originally proposed by Nicoll, mutational analysis is entirely consistent with the concept, for primate specificity of binding by the hGHR. Our four-way fold changes in whole cell affinity for each hormone (relative to wild type hGH). Data are taken from Tables I–IV.

The complementary mutation in the rbGHR (L43R) sets up a greater than 200-fold increase in binding affinity (4), presumably because of the loss of the species determinants, it must be said that pGH is poorly effective even against the non-primate rabbit receptor (Table III), so the residual 17-fold loss in affinity may be a result of generally poorer binding of pGH to GH receptors.

The data presented here provide strong support both for our original postulate (8, 20) that receptor Arg-43 is a primate specificity determinant, and for the results of Souza et al. (9), wherein mutation of Leu-43 of mouse or bovine receptor to arginine resulted in primate type specificity. Why then, is bovine GH binding to intact cells only slightly decreased in affinity when tested against the L43R rbGHR (Table IV and Ref. 8)? As can be seen from Tables III and IV, bGH binds very poorly to the solubilized L43R rabbit receptor (200-fold lower affinity than for wild type rbGHR), yet it is unusual in that its affinity in the whole cell assay is essentially unchanged relative to wild type rabbit receptor. The biopotency falls between these extremes (29-fold lower than against wild type rbGHR). These apparently contradictory observations may be resolved by the unusually high site 2 affinity reported for bGH binding to its receptor (21), which would strongly favor formation of the ternary complex over the 18 h at 4 °C required to reach binding equilibrium. That bGH binding to primate receptor is restricted by the same mechanism as pGH is apparent in Tables I and II, where substitution of leucine for Arg-43 of the human receptor is able to increase binding 90-fold and biopotency 65-fold.

The interactions involving human receptor Arg-43 and Asp-171 of hGH are shown in Fig. 3A, and those of rabbit receptor Leu-43 and His-170 of pGH, homology modeled from the human coordinates, are shown in Fig. 3B. It can be seen that the human receptor-hormone interaction is stabilized by a salt bridge between receptor Arg-43 and Asp-171 and a hydrogen bond between Arg-43 and hormone Thr-175. The alkylation of Arg-43 also shares a hydrophobic interaction with Trp-169.

Fig. 2. Correlation plots of affinity against bioactivity. Panel A, fold changes in whole cell affinity for each hormone (relative to wild type hGH) plotted against fold changes in proliferative ability (relative to wild type hGH). Data are taken from Tables I–IV. Panel B, fold changes in site 1 affinity for each hormone (relative to wild type hGH) plotted against fold changes in proliferative ability (relative to wild type hGH). Data are taken from Tables I–IV.

**DISCUSSION**

This is the first comprehensive study of the structural basis for primate specificity of binding by the hGHR. Our four-way mutational analysis is entirely consistent with the concept, originally proposed by Nicoll et al. (15), that a hindrance determinant is responsible for restricting receptor binding to primate growth hormones. As can be seen in Fig. 3, in the case of non-primate hormones, the steric hindrance resulting from the incompatibility of histidine at hormone residue 170 and arginine at receptor residue 43 is presumably exacerbated by repulsive interactions between these basic residues. In the case of pGH, substitution of this single histidine with aspartate results in a greater than 200-fold increase in binding affinity and potency, to around 1/20 of hGH (Table I). Conversely, substitution of receptor Arg-43 with non-primate leucine in the hGHR allows pGH to bind, again with around 5% of the affinity and potency of hGH (Table II). With the R43L hGHR mutant, substitution of His-170 with aspartate now results in only a minor increase in site 1 affinity and no change in biopotency. The complementary mutation in the rbGHR (L43R) sets up primate type specificity in that pGH binds and acts weakly, and only “humanized” pGH (H170D) is able to bind and act reasonably effectively on this receptor mutant (Tables III and IV). In the light of these findings, it is interesting to note that ovine and bovine placental lactogens, which are unique among non-primate hormones in being able to bind to the human receptor with high affinity (16) despite only a 25% homology to hGH, possess serine at equivalent position 171, unlike any other GHs.

With other class 1 cytokine receptors displaying species restriction in binding (e.g., the leukemia inhibitory factor receptor (17) or the granulocyte-macrophage colony-stimulating factor receptor (18)) mutagenesis has revealed that several residues are responsible. Although the evidence presented here supports a central role for His-170 in determining binding specificity, other species-specific determinants may reside in the unstructured loop between helices 1 and 2, based on the homolog scanning mutagenesis study of Cunningham et al. (19). In that study, while substituting pGH residues 164–191 into hGH abolished binding to the hGHR binding domain, substitution of pGH residues 54–74 resulted in a 17-fold decrease in affinity. Although these data can be interpreted as indicating other species determinants, it must be said that pGH is poorly effective even against the non-primate rabbit receptor (Table III), so the residual 17-fold loss in affinity may be a result of generally poorer binding of pGH to GH receptors.

The data presented here provide strong support both for our original postulate (8, 20) that receptor Arg-43 is a primate specificity determinant, and for the results of Souza et al. (9), wherein mutation of Leu-43 of mouse or bovine receptor to arginine resulted in primate type specificity. Why then, is bovine GH binding to intact cells only slightly decreased in affinity when tested against the L43R rbGHR (Table IV and Ref. 8)? As can be seen from Tables III and IV, bGH binds very poorly to the solubilized L43R rabbit receptor (200-fold lower affinity than for wild type rbGHR), yet it is unusual in that its affinity in the whole cell assay is essentially unchanged relative to wild type rabbit receptor. The biopotency falls between these extremes (29-fold lower than against wild type rbGHR). These apparently contradictory observations may be resolved by the unusually high site 2 affinity reported for bGH binding to its receptor (21), which would strongly favor formation of the ternary complex over the 18 h at 4 °C required to reach binding equilibrium. That bGH binding to primate receptor is restricted by the same mechanism as pGH is apparent in Tables I and II, where substitution of leucine for Arg-43 of the human receptor is able to increase binding 90-fold and biopotency 65-fold.

The interactions involving human receptor Arg-43 and Asp-171 of hGH are shown in Fig. 3A, and those of rabbit receptor Leu-43 and His-170 of pGH, homology modeled from the human coordinates, are shown in Fig. 3B. It can be seen that the human receptor-hormone interaction is stabilized by a salt bridge between receptor Arg-43 and Asp-171 and a hydrogen bond between Arg-43 and hormone Thr-175. The alkylation of Arg-43 also shares a hydrophobic interaction with Trp-169. In addition, there may be a weak hydrogen bond between Asp-171 and the Nε1 of receptor Trp-104. Because of the extensive network of interactions involving Arg-43, it is to be expected that alanine substitution would result in a substantial decrease in affinity, and a loss in binding free energy of around 2 kcal/mol was reported in Ref. 5. However, as can be seen from Table II, leucine substitution results in no significant change in site 1 affinity, leading to the conclusion that guanido headgroup interactions are not as important as the hydrophobic interaction with receptor Trp-169, which presumably still occurs with leucine at residue 43. It has been reported that alanine substitution of Asp-171 results in an approximately 4-fold loss in affinity (4), presumably because of the loss of the
Species Specificity of Growth Hormone Binding

**Fig. 3.** Side chain interactions in the critical species-determining region of GH/GHR. Panel A, hGH side chains D171 and T175 interacting with hGHR side chains R43, W104, and W169 taken from the hGH(hGHBP)_2 crystal structure (3). Distances between particular atoms are as follows: Arg-43 N to Thr-175 Oy1 = 3.30 Å, Arg-43 N to Asp-171 Oy1 = 3.30 Å, and Trp-104 Ne1 to Asp-171 Oe2 = 3.1 Å, indicating likely hydrogen bonds and salt bridges. Panel B, pGH side chains His-170 and Thr-174 interacting with rbGHR side chains Leu-43, Trp-104, and Trp-169 taken from the homology-modeled structure based on the pGH(hGHBP)_2 crystal structure (10). The distance between Trp-104 Nε1 and His-170 Nε1 is 3.2 Å, and the angle between Trp-104 Nε1, Trp-104 Hε1, and His-170 Nε1 is 144.4°, indicating a possible hydrogen bond. Atoms are colored as follows: carbon, green; oxygen, red; nitrogen, blue. Panel C, hGH side chain Asp-171 and hGHR side chain Arg-43 (both red) from hGH(hGHBP)_2 crystal structure (3) superimposed over pGH side chain His-170 and rbGHR side chain Leu-43 (both blue) from the homology-modeled pGH/rbGHR structure (10) showing the unfavorable interaction that would occur between pGH His-170 and hGHR Arg-43 (distance between His-170 Ne2 and Arg-43 Nε1 would be 1.98 Å).

In the current study we have elected to use three kinds of assays to investigate the GH-receptor interaction. The solubilized receptor binding assay, carried out in the presence of the dimerization-blocking antibody MAB 5 (14), provides a measure of site 1 affinity for the intact receptor. The whole cell binding assay, carried out at 4 °C to prevent internalization, is intended to provide a measure of formation of the ternary complex and as such includes avidity and membrane geometry effects (22). The proliferation bioassay, performed on the same cell lines as for the two binding assays, provides accurate biopotency data with full-length human and rabbit receptors. Inspection of Tables I–IV shows that generally the whole cell binding affinity is closely related to the bioassay value in terms of rank order and magnitude (Fig. 2A, correlation coefficient 0.99, p < 0.001, excluding bGH with L43R rGHR), whereas site 1 affinity does not correlate with biopotency (Fig. 2B).

With D171H hGH, large changes in site 1 affinity do not manifest as large changes in biopotency, either with the human or rabbit receptor. This may indicate a strong propensity for hGH to form site 2 interactions. The pGH-hGH receptor interaction on the other hand, does not show a large difference between site 1 affinity and whole cell biopotency, which could be a result of weak site 2 interactions. With the rabbit receptor, rank order and magnitude of whole cell affinity and biopotency again correlate well, except in the case of bGH and the L43R rbGHR, for the reasons discussed above. Based on the results with wild type pGH binding to the rabbit receptor (i.e. a sizable disparity between site 1 affinity and whole cell affinity), one could propose that site 2 interactions here are stronger than for the R43L human receptor. For reasons that are not clear, this disparity is not as marked with the H170D pGH analog. Further high performance liquid chromatography studies with purified human and rabbit GHBPs are needed to provide a quantitative basis for these proposals, but it would appear that this analog is not as effective as wild type in inducing trimer formation. What is clear is that substantially decreasing the affinity of hGH (50-fold) has little effect on biopotency, whereas with pGH a marked effect on biopotency is seen with altered affinity. This is concordant with the finding of
that large changes in site 1 affinity of hGH do not manifest
as large changes in biopotency using a GH/granulocyte colony-
stimulating factor receptor chimera-expressing myeloma line.
However, our findings are also consistent with our previous
finding (11) that changes in site 1 binding determinants of pGH
do result in substantial changes in biopotency, such that a
5-fold increase in affinity and biopotency over wild type pGH
was seen with the C181S(del 183–191) site 1 mutant. Accord-
ingly, it is possible to improve substantially the biopotency of
pGH at least, by increasing site 1 affinity.

Given the incompatibility of His-170 and receptor Arg-43 and
its compatibility with leucine (Fig. 3C), the likely evolutionary
scenario is a single base mutation of the histidine to aspartate,
followed by single base change of receptor leucine to arginine.
The fact that aspartate is present at this position in all five
primate GH genes indicates that the initial event must have
occurred very early in primate evolution. Based on Tables I and
II, there does not appear to be any particular advantage in
selection of arginine at position 43 relative to leucine other
than a 2-fold increase in proliferative potency. In the the re-
ceptor 2 binding site, Arg-43 forms a hydrogen bond with hGH
Asn-12 (3), although the mutagenic analysis of Ref. 14 shows
that this provides minimal binding energy. The prolactin re-
ceptor has lysine in the equivalent position, but this is not a
binding element (23).

In conclusion, it has been possible to create a non-primate
hormone with the ability to bind to and activate the human
receptor by mutating one residue. The structural basis for
primate specificity resides in the incompatibility of this residue
with a hindrance determinant on the primate receptor, evident
in the crystal structure of the hormone-receptor complex. It is
salutary that although the strongest positive interaction in
hGH contributes 20-fold to the affinity (24), a single unfavor-
able interaction can reduce the affinity of pGH more than
300-fold and its biopotency more than 160-fold.

REFERENCES
1. Wells, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1–6
2. Takahashi, Y., Kaji, H., Okimura, Y., Goji, K., Abe, H., and Chihara, K. (1996)
   N. Engl. J. Med. 334, 432–436
3. De Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) Science 255, 306–312
4. Cunningham, B. C., and Wells, J. A. (1990) J. Mol. Biol. 234, 554–563
5. Clackson, T., and Wells, J. A. (1995) Science 267, 383–386
6. Cunningham, B. C., and Wells, J. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88,
   3407–3411
7. Lauterio, T. J., Trivedi, B., Kapadia, M., and Daughaday, W. H. (1988) Comp.
   Biochem. Physiol. 91, 15–19
8. Gebius, K. S., Rowlinson, S. W., Barnard, R., Mattick, J. S., and Waters, M. J.
   (1992) J. Mol. Endocrinol. 9, 213–222
9. Souza, S. C., Frick, G. P., Wang, X., Kopchick, J. J., Lobo, R. B., and Goodman,
   H. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 959–963
10. Rowlinson, S. W., Barnard, R., Bastiras, S., Robins, A. J., Senn, C., Wells,
    J. R. E., Brinkworth, R., and Waters, M. J. (1994) Biochemistry 33, 11724–11733
11. Rowlinson, S. W., Barnard, R., Bastiras, S., Robins, A. J., Brinkworth, R., and
    Waters, M. J. (1993) J. Biol. Chem. 268, 11724–11733
12. Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and
    Wells, J. A. (1992) Science 256, 1677–1680
13. Cunningham, B. C., Ultsch, M., Abraham, M., DeVos, A. M., Mulkerrin, M. G.,
    Clauser, K. R., and Wells, J. A. (1991) Science 254, 821–825
14. Nicoll, C. S., Mayer, G. L., and Rassu, S. M. (1986) Endocr. Rev. 7, 169–203
15. Avital, R., Holder, A. T., Preece, M. A., and Ivanov, V. A. (1986) J. Endocrinol. 30,
    381–388
16. Layton, M. J., Owezarek, C. M., Metcalfe, D., Clark, R. L., Smith, D. K.,
    Trouche, H. B., and Nicola, N. A. (1994) J. Biol. Chem. 269, 18467–18473
17. Shanafeld, A. B., Johnson, K. E., and Kastel, R. A. (1991) J. Biol. Chem. 266,
    13804–13810
18. Cunningham, B. C., Jhurani, P., Ng, P., and Wells, J. A. (1988) Science 243,
    1359–1360
19. Barnard, R., Rowlinson, S. W., and Waters, M. J. (1989) J. Biol. Chem. 266,
    556–567
20. Staden, N. R., Byatt, J. C., and Krivi, G. G. (1993) J. Biol. Chem. 268,
    18467–18473
21. Philo, J. S., Aoki, K. H., Arakawa, T., Narhi, L. O., and Wen, J. (1996)
    Biochemistry 35, 16831–16836
22. Rozakis-Adcock, M., and Kelly, P. A. (1992) J. Biol. Chem. 267, 7428–7433
23. Cunningham, B. C., and Wells, J. A. (1989) Science 244, 1081–1085