Functional Promiscuity of Squirrel Monkey Growth Hormone Receptor Toward both Primate and Nonprimate Growth Hormones

Soojin Yi,* Bryan Bernat,†[1] Gábor Pál,† Anthony Kossiakoff,† and Wen-Hsiung Li*

*Department of Ecology and Evolution and †Department of Biochemistry and Molecular Biology, University of Chicago

Primate growth hormone (GH) has evolved rapidly, having undergone ~30% amino acid substitutions from the inferred ancestral eutherian sequence. Nevertheless, human growth hormone (hGH) is physiologically effective when administered to nonprimate mammals. In contrast, its functional counterpart, the human growth hormone receptor (hGHR), has evolved species specificity so that it responds only to Old World primate GHs. It has been proposed that this species specificity of the hGHR is largely caused by the Leu → Arg change at position 43 after a prior His → Asp change at position 171 of the GH. Sequence analyses supported this hypothesis and revealed that the transitional phase in the GH:GHR coevolution still persists in New World monkeys. For example, although the GH of the squirrel monkey has the His → Asp substitution at position 171, residue 43 of its GHR is a Leu, the nonprimate residue. If the squirrel monkey truly represents an intermediate stage of GH:GHR coevolution, its GHR should respond to both hGH and nonprimate GH. Also, if the emergence of species specificity was a result of the selection for a more efficient GH:GHR interaction, then changing residue 43 of the squirrel monkey growth hormone receptor (smGHR) to Arg should increase its binding affinity toward higher primate GH. To test these hypotheses, we performed protein-binding assays between the smGHR and both human and rat GHs, using the surface plasmon resonance methodology. Furthermore, the effects of reciprocal mutations at position 43 of human and squirrel monkey GHRs are measured for their binding affinities toward human and squirrel monkey GHs. The results from the binding kinetic assays clearly demonstrate that the smGHR is in the intermediate state of the evolution of species specificity. Interestingly, the altered residue Arg at position 43 of the smGHR does not lead to an increased binding affinity. The implications of these results on the evolution of the GH:GHR interaction and on functional evolution are discussed.

Introduction

The mammalian pituitary growth hormone (GH) has been studied extensively in various disciplines of biological research (Wells 1994; Kossiakoff 1995; Li 1997, p. 180; Liu et al. 2001). Much information has come from studying the interaction of the human growth hormone (hGH) with the extracellular domain (ECD) of the human growth hormone receptor (hGHR) (also called the GH-binding protein but referred to as GHR-ECD in this report), which is found freely circulating in serum and is able to bind hGH with the same affinity as does the full-length receptor (Fuh et al. 1990). The biologically active ternary complex is formed from one GH molecule and two GHRs through a sequential process of high-affinity site 1 binding followed by site 2 binding (De Vos, Ultsch, and Kossiakoff 1992; Wells 1994).

The GH and the GHR also provide an opportunity to understand the coevolution of two proteins. Wallis (1994) compiled the evolutionary rates of GHs from various mammalian taxa to show that, in general, GH evolves at a slow basal rate, but significant acceleration in evolutionary rate occurred in two lineages, one leading to the primates (human and Rhesus monkey) and the other to the artiodactyls (Wallis 1994). The acceleration seen in the primate lineage is exceptionally high (Li 1997). It has been proposed that positive selection may

be a driving force for the observed acceleration (Wallis 1996; Liu et al. 2001; Wallis, Zhang, and Wallis 2001). The acceleration was particularly conspicuous at "functionally important" sites (Liu et al. 2001), which were inferred from functional epitope mapping of the hGH·(hGHR-ECD)$_2$ complex (Cunningham and Wells 1989, 1991, 1993), as well as the tertiary structure of the hGH and human prolactin receptor complex (Somers et al. 1994).

Among these functionally important sites, one pair of residues is of particular interest with regard to the origin of species specificity of the hGHR. In general, protein hormones from nonprimates are active in humans. However, human and Rhesus monkey GHRs are unresponsive to GHs from nonprimate mammals (Carr and Friesen 1976; Peterson and Brooks 2000). In contrast, the hGH is fully functional when administered to other mammalian species. This pattern of high specificity and cross-reactivity is not well understood at either the biochemical or the evolutionary level. On the basis of the sequence comparisons, Souza et al. (1995) proposed that the interaction between residue 171 of the GH (GH171Asp in human, which is equivalent to GH170His in nonprimates) and residue 43 of the GHR (GHR43Arg in human and GHR43Leu in nonprimates) is the major determinant of the hGHR species specificity. At the hGH:hGHR site 1 interface, GH171Asp and GHR43Arg are known to form a strong salt bridge and are, therefore, assumed to be essential for the formation of the hormone-receptor complex (De Vos, Ultsch, and Kossiakoff 1992; see fig. 1). Among the residues that are known to form favorable salt bridges and hydrogen bonds at the site 1 interface, these two are the only pair of structurally complementary residues that differ at
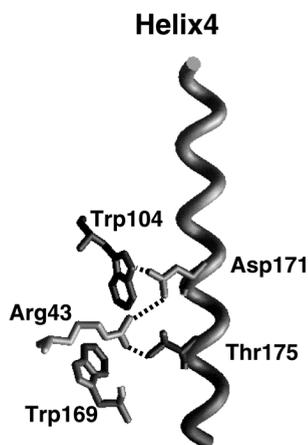
Helix4

Fig. 1.—Structural interactions surrounding hGHR43Arg at the site 1 interface. The hGH helix 4 side chains of Asp171 and Thr175 interacting with the hGHR side chains of Arg43 and Trp104.

both positions between primates and nonprimates (see table 1).

Evolutionary analyses of GH and GHR sequences from simians and prosimians showed that the substitution GH171His → GH171Asp occurred in the lineage leading to the common ancestor of Platyrrhines and Catarrhines (Liu et al. 2001; Wallis, Zhang, and Wallis 2001), followed by the substitution GHR43Leu → GHR43Arg in the common ancestor of the Old World monkeys (Liu et al. 2001). Notably, Liu et al. (2001) reported the presence of an ''intermediate'' stage; the two New World monkey species studied, the squirrel monkey and the spider monkey, possess the primate-type GH (Asp171), whereas their GHRs still have the nonprimate-type Leu at residue 43. If the compatibility between GH171 and GHR43 is truly a major determinant of the binding specificity, then the GHR of the New World monkey species represents a functionally intermediary status. Therefore, GHRs from these species should bind to both primate and nonprimate GHs. They also provide a chance to examine whether a functionally intermediate stage of evolution reflects an intermediate stage of natural selection, in other words, whether the GHR43Leu → GHR43Arg substitution has any selective advantage, i.e., if the evolution of the hGHR species specificity was a result of positive selection.

In this study we investigated the range of binding affinities of squirrel monkey growth hormone receptor (smGHR) by conducting in vitro binding assays between the squirrel monkey growth hormone receptor ECD (smGHR-ECD) and the GHs from human, squirrel monkey, and rat (a nonprimate outgroup). The surface plasmon resonance (SPR) measurement technique was used to measure the kinetic rate constants and the corresponding binding affinities. Our study suggests that the smGHR-ECD does represent an intermediary form of species specificity. To further test whether this is caused by the compatible interaction between GH171 (GH170 in nonprimates) and GHR43, we engineered and produced human and squirrel monkey GHR-ECDs with converse amino acids at residue 43. By this, we were able to alter smGHR-ECD to acquire species specificity,

Table 1

Alignment of the Sites Known to be Involved in the Site 1 Interaction of hGH and hGHR from the Three Species Used in this Study

GH				GHR			
Residue	Rat	Squirrel Monkey	Human	Residue	Rat	Squirrel Monkey	Human
10	Phe	Leu	Phe	*43*[171, 175]	*Leu*	*Leu*	*Arg*
18	Gln	His	His	44	Glu	Glu	Glu
25	Ala	Phe	Phe	70	Arg	Arg	Arg
29	Lys	Gln	Gln	71	Arg	Arg	Arg
41[127]	*Arg*	*Lys*	*Lys*	73	Ala	Thr	Thr
45	Ile	Leu	Leu	74	His	Gln	Gln
46[120]	*Gln*	*Gln*	*Gln*	75	Glu	Glu	Glu
48	Ala	Pro	Pro	76	Trp	Gly	Trp
54	Phe	Phe	Phe	80	Trp	Trp	Trp
56	Glu	Glu	Glu	98	Ser	Ser	Ser
58	Ile	Ile	Ile	102	Ser	Ser	Ser
61[103]	*Pro*	*Pro*	*Pro*	*103*[61]	*Ile*	*Ile*	*Ile*
64	Lys	Lys	Arg	*104*[168] . . .	*Trp*	*Trp*	*Trp*
65	Glu	Lys	Glu	105	Ile	Ile	Ile
68	Gln	Gln	Gln	106	Pro	Pro	Pro
167[127]	*Lys*	*Arg*	*Arg*	*120*[46]	*Glu*	*Glu*	*Glu*
168[104]	*Lys*	*Lys*	*Lys*	121	Lys	Lys	Lys
171[43]	**His**	**Asp**	**Asp**	124	Thr	Ser	Ser
172	Lys	Lys	Lys	126	Asp	Asp	Asp
174	Glu	Glu	Glu	*127*[41, 167] . .	*Glu*	*Gln*	*Glu*
175[43]	*Thr*	*Thr*	*Thr*	164	Asp	Asp	Asp
178[165]	*Arg*	*Arg*	*Arg*	*165*[178] . . .	*Val*	*Ile*	*Ile*
179	Val	Ile	Ile	166	Leu	Gln	Gln
182	Cys	Cys	Cys	167	Lys	Lys	Lys
183	Arg	Arg	Arg	169	Trp	Trp	Trp
185	Val	Val	Val	171	Ile	Val	Val
				195	Thr	Thr	Thr
				216	Gln	Gln	Gln
				217	Arg	Arg	Arg
				218	Ser	Lys	Asn
				219	Phe	Ser	Ser

Note.—Residues involved in salt bridges and hydrogen bonds at the hGH: hGHR-ECD site 1 interface (De Vos, Ultsch, and Kossiakoff 1992) are shown in italic, with the complementary residues in superscript. The structurally complementary residues GH171 and GHR43 are shown in bold. The rest of the table represents the residues comprising the interface for site 1 binding as shown by alanine-scanning mutageneses of hGH and hGHR (Cunningham and Wells 1989, 1991, 1993; Clackson and Wells 1995; Clackson et al. 1998).

whereas hGHR-ECD with Leu at position 43 gained an affinity toward rat GH, thereby losing its species specificity. In contrast to these significant effects of mutations at residue 43 of primate GHRs on the interactions with the nonprimate GH, the same mutations have very little effect on the binding affinities toward primate GHs. This demonstrates an example of a single mutation in a protein hormone that has very different functional consequences on its interactions with receptors from distinct phylogenetic groups.

Materials and Methods
Oligonucleotide-Directed Mutagenesis

The cDNA clones for the squirrel monkey (*Saimiri boliviensis*) growth hormone (smGH) and the smGHR-ECD were obtained from a previous study (Liu et al. 2001). Mutations were introduced via oligonucleotide-directed mutagenesis (Kunkel, Roberts, and Zakour 1987) using CJ236 (dut−, ung−) *Escherichia coli* cells from Bio-Rad (Hercules, Calif.) and uracil-containing

Table 2
Primers Used for ph-Expression Vector Construction

Expression Plasmid	Primers
hGHR-ECD 43Leu237Cys........	hGHrLeu43mut (for Kunkel mutagenesis) 5′-TGC CGT TCA CCT GAG CTG GAG ACT TTT TCA TGC-3′
smGH........................	5Nsi1smGH (for 5′ addition of Nsi1 site) 5′-AAA AAA TGC ATT CCC AAC AAT TCC CTT ATC CAG GCT-3′ 3Not1smGH (for 3′ addition of Not1 site) 5′-AGA ACA GTA AAA CGC GGC CGC GGG CTA GAA GCC ACA-3′
smGHR-ECD 237Cys............	5Nsi1smGHR (for 5′ addition of Nsi1 site) 5′-AAA AAT GCA TCT TCT AAG GAG CCT AAA TTC AC-3′ 3Not1smGHR (for 3′ addition of Not1 site) 5′-AAA CGC GGC CGC TAC TGG CAC ATC TGA GGA AGT-3′
smGHR-ECD Leu43Arg237Cys ...	smGHrArg43mut (for Kunkel mutagenesis) 5′-TGC CGT TCA CCT GAG CGC GAG ACT TTT TCA TGC-3′

single-stranded DNA produced using M13-VCS helper phage from Stratagene (La Jolla, Calif.). Custom oligonucleotides carrying the designed mutation were purchased from Integrated DNA Technologies Inc. (Coralville, Iowa). Following Clackson et al. (1998), truncated forms of GHR-ECDs (amino acid residues 29–238) were expressed and isolated for binding studies. A Ser237Cys mutation was introduced into GHR-ECDs so that the constructs could be attached to the biosensor chip (see *Biosensor Surface Preparation* for more detail). All mutations were confirmed at the nucleotide level by sequencing on an ABI 377 sequencer. The oligonucleotide sequences used to introduce mutations are shown in table 2.

Construction of Expression Plasmids

The expression vector used for this study was from Genentech Inc. (South San Francisco, Calif.) (Fuh et al. 1990). All constructs discussed in this report were made using the phGHr expression plasmid (Fuh et al. 1990). NsiI and NotI restriction sites were added to the 5′ and 3′ ends of each insert by PCR. The PCR primers used are shown in table 2. At least eight single colonies per construct were picked and analyzed for their plasmids by sequencing the entire inserts. For general cloning purpose, the JM109 strain of *E. coli* from Invitrogen (Carlsbud, Calif.) was used. For expression, the proteinase-deficient BL21 strain of *E. coli* from Stratagene was used.

Expression and Purification of Periplasmic Proteins

BL21 cells were transformed with each expression plasmid. From the resulting plates, a single colony was picked and inoculated in the Luria-Bertani media containing 60 µg/ml ampicillin in 1/100th volume of the final culture. The cells were grown for 4 h at 37°C, then washed with and transferred into a larger volume of low-phosphate AP-5 media to induce the alkaline phosphatase promoter–directed transcription of the recombinant proteins (Fuh et al. 1990). The optimized growth conditions for all the GHR-ECD variants are at 20°C for 28 h, with shaking at 225 rpm. Expression plasmid for smGH was grown at 32°C for 11 h. Cells were then harvested to be frozen at −80°C. Periplasmic proteins were extracted through repeated freezing-thawing steps in 1/100 volume of the original culture of 10 mM Tris-Cl (pH 8.0). Whether the protein of expected size was present or not in the periplasmic protein fraction was checked from this stage by SDS polyacrylamide gel electrophoresis (PAGE) or by Western blotting (or both), using the anti-hGH (catalog number E45600M, Bio-Design International, Kennebunk, Me.) for GHs and MAb against hGHR (product number 7005B, American Diagnostica Inc., Greenwich, Conn.). The supernatant was then ammonium sulfate precipitated to be resuspended into 3 ml of 10 mM Tris-HCl, pH 8.0, buffer and then further processed according to the different purification procedures discussed subsequently.

Fast Protein Liquid Chromatography Purification of hGH

The ammonium sulfate–precipitated periplasmic protein fraction containing hGH was desalted on a PD-10 column (Amersham Pharmacia Biotechnology, Piscataway, NJ) and applied onto a Resource Q column (Amersham Pharmacia Biotechnology) equilibrated with 10 mM Tris-Cl (pH 8.0). The hormone was then eluted by a 0- to 300-mM NaCl gradient using a Fast Protein Liquid Chromatography apparatus (Varian Prostar model 210). The resulting fractions were concentrated on a Centricon 10k filtering unit (Amicon) and analyzed on SDS-PAGE, reverse-phase HPLC (Shimadzu LC10AD VP system with a Vydac Protein C4 column), and mass spectrometry (PE Sciex API 150Ex). The concentrations of all samples were determined by absorbance at 280 nm. Extinction coefficients were derived from the tryptophan and tyrosine content (for example, ε_{280} hGH = 16,200/[M cm], ε_{280} hGHR-ECD =55,000/[M cm]). The

recombinant rat GH used in this study was obtained from the National Hormone and Peptide Program (by courtesy of Dr. A. F. Parlow, http://www.humc.edu/hormones).

Affinity Chromatography for the Isolations of GHR-ECDs

Each ammonium sulfate–precipitated periplasmic protein fraction containing GHR-ECD was applied to a hGH affinity column (Fuh et al. 1990), which was washed and equilibrated in 20 mM Tris-HCl, pH 8.0. The 2-ml column was then washed with the same buffer containing 10 mM $MgCl_2$ and finally with 1.0 M NaCl. The specifically bound proteins were then eluted with 2.5 ml 4.5 M $MgCl_2$. The eluted proteins were immediately desalted on a PD-10 column (Amersham Pharmacia Biotechnology) in 10 mM sodium phosphate buffer, pH 7.4, and then further analyzed as described above.

Affinity Chromatography for smGH Isolation

To purify smGH, an smGHR-ECD affinity column was constructed. First, ~5 mg of smGHR-ECD237Cys from a 10-liter culture was purified using an hGH affinity column. Because this GHR-ECD contained 237Cys mutation, it was glutathione modified on the unpaired cysteine by *E. coli*. After removing the glutathione modification to free the thiol residue (see *Biosensor Surface Preparation* for the detailed protocol), the purified smGHR-ECD was biotinylated using the EZ-Link® PEO-Maleimide–activated biotin (Pierce, Rockford, Ill.) and then coupled to immobilized streptavidin–containing beads (UltraLink® immobilized streptavidin plus, Pierce). We were able to obtain >95% pure smGH using this column. Preliminary binding experiments with hGHR-ECD using the pure smGH obtained from this column led to an observation that the smGH binds to hGHR-ECD with high affinity. After this, we constructed a higher-capacity hGHR-ECD affinity column for the purification of a larger amount of smGH, using >15 mg of hGHR-ECD immobilized through random amine coupling using CNBr-activated Sepharose beads (Amersham Pharmacia Biotechnology). The smGH was then purified following the same affinity column purification steps described earlier and desalted in PBS (pH 7.4).

Preparation of GHR-ECDs for Immobilization

As mentioned earlier, a point mutation Ser237Cys was introduced in the receptor. The Ser237Cys mutation does not affect hormone binding and allowed us to immobilize the GHR-ECDs in a homogeneously oriented manner, using a thiol-disulfide exchange coupling method. In all cases, the extra unpaired cysteine residue on the receptor introduced by this mutation became covalently modified in *E. coli* by glutathione. This modification was removed by a 30-min treatment in 1 mM dithiothreitol (DTT) on ice for the Ser237Cys mutants of hGHR-ECD and hGHR-ECD43Leu. The Ser237Cys-containing smGHR-ECD and smGHR-ECD43Arg were treated in a milder reducing reagent, 7.5 mM β-mercaptoethanolamine for 15 min at 37°C in the presence of 50 mM EDTA.

Biosensor Surface Preparation

BIAcore Pioneer C-1 sensor chips were used for the analyses. Reagents for this, *N*-ethyl-*N'*-(*s*-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydrosuccinimide (NHS), and 2-(2-pyridinyldithio) ethaneamine hydrochloride (PDEA), were purchased from Biacore (Uppsala, Sweden). First, the surface of the Pioneer C-1 chip was treated with 15 μl of 100 mM Na-glycinate and 0.3% Triton-X solution (pH 12.0) and was then equilibrated with degassed HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4). Then, the surface carboxyl groups were activated with a 0.05 M NHS–0.2 M EDC solution. Activated thiol groups were introduced by a 40-μl injection of 80 mM PDEA (in 0.1 M sodium carbonate buffer pH 8.5). This step was followed by manual injection of the Ser237Cys-containing GHR-ECDs of 0.1-mg/ml concentration in 10 mM sodium acetate pH 4.5 buffer. The excess reactive thiol compound was quenched by a 30-μl injection of 50 mM glutathione. The control surface was activated and blocked as mentioned before but had no protein immobilized. The same HBS buffer, as mentioned before, was used for all the measurements with the flow rate of 50 μl/min.

SPR Measurements and Analyses of Binding Kinetics Data

A BIAcore2000 measures the refractive index near the sensor surface by SPR (see Fisher et al. 1994 for an application of this technology). We injected twofold serial dilutions of the GH solutions into cuvettes containing the analysis chip. Buffer-only injection representing 0 nM analyte was also included and subtracted from all the sensorgrams in the same set to minimize the noise introduced by the experimental procedure. All measurements were repeated at least four times to ensure reproducibility. On average, two independent measurements from two chips were performed to assess the interactions.

Because of the short and rigid linker of the C-1 chip, the immobilized receptor molecules cannot interact with each other. Thus, GH binding specifically through site 1 can be analyzed without the complication of subsequent interactions through site 2 (Bernat et al., unpublished data). The kinetics of the site 1 GH:GHR-ECD interaction can, therefore, be accurately described by a simple bimolecular or Langmuir interaction:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$
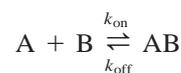
where k_{on} is the association rate constant and k_{off} the dissociation rate constant. Analyzing the kinetics of the binding interactions, we determined both k_{on} and k_{off} values and ultimately the corresponding equilibrium dissociation constant ($K_d = k_{off}/k_{on}$) value.

Using the sensorgrams from analytes of different concentrations, a global fit of both association and dissociation rate constants for each interaction was generated. The data were analyzed using the BIA evaluation 3.0 software (Biacore), applying the simple 1:1 (Langmuir) binding model for the fitting.

Results

Expression and Purification

Typical yields for hGH, hGHR-ECD, and hGHR-ECD43Leu were at 4 mg recombinant protein purified from 1 liter of the culture. Expression of smGH, smGHR-ECD, and smGHR-ECD43Arg was much lower, resulting in less than 1 mg purified protein from 1 liter of the culture. After affinity chromatography, the samples were 95% pure, as judged by HPLC analysis. We confirmed the presence of all the desired mutations by measuring the mass differences caused by differently encoded amino acids using mass spectrometry. In the case of smGH, the prolonged time of expression resulted in a mass of 18 units above the theoretical mass, presumably because of an additional water molecule resulting from the cleavage of one of the peptide bonds. The cleaved and intact hormones have practically the same binding characteristics (data not shown). When the cells were grown over 25 h, 100% of the proteins were in cleaved form. Reducing the growth time to less than 12 h successfully prevented this cleavage. Only data for the intact forms were included in this paper.

All Ser237Cys variants were modified by glutathione at the unpaired cysteine residue during the expression. This modification was successfully removed by DTT without affecting the native disulfides of the protein for 237Cys mutants of hGHR-ECD and hGHR-ECD43Leu. This was not the case for smGHR-ECD and its variant. The same condition turned out to be too harsh for the 237Cys smGHR-ECD variant, leading to reduction of the native disulfide bonds and ultimate denaturation of the protein. Despite the small number of amino acid sequence differences between human and squirrel monkey GHRs, the stability and reactivity of their disulfides are measurably different, suggesting subtle tertiary structure differences. A milder reducing reagent β-mercapthoethanolamine was used to empirically determine an optimal treatment condition. A 20-min treatment with 7.5 mM β-mercapthoethanolamine solution at 37°C with the presence of 50 mM EDTA was effective and safe for the removal of the glutathione modification.

Determination of Binding Kinetics

SPR is a method capable of measuring the binding interactions between molecules by using the changes in refractive index to measure the mass changes of molecules bound to the analysis chip. These changes were reported as "response units (RUs)." The RU values are linearly proportional to the amount (mass) of the analytes binding to the ligands. A typical sensorgram is composed of two characteristic phases. In the first (association) phase, the GH is injected above the GHR-ECD–containing surface, allowing the association of the GH to the immobilized GHR-ECD. However, simultaneous dissociation of the GH from the resulting GH:GHR-ECD complex also occurs in this phase. A sufficiently long injection time leads to a steady-state response level characterized by equal rates of association of the GH to GHR-ECD and of dissociation of the GH from the GH:GHR-ECD complex. In the second (dissociation) phase, instead of the GH, only the running buffer is injected. In this phase only the dissociation of the GH from the previously formed GH:GHR-ECD complexes occurs because there is no further supply of GH injected over the surface.

Different Species Specificities of Human and Squirrel Monkey GHR-ECDs

Figure 2A shows the sensorgrams generated from the responses of hGHR-ECD237Cys to the three different GHs: hGH, smGH, and rat GH. The interaction between hGH and hGHR-ECD has a K_d = 3.5 nM (table 3). Interestingly, the smGH binds to the hGHR-ECD with approximately the same affinity as hGH does (K_d = 3.3 nM). As expected, the rat GH does not bind to the hGHR-ECD.

Figure 2B shows the sensorgrams from the binding of smGHR-ECD237Cys to three different hormones. The hGH and the smGH bind to the smGHR-ECD with virtually identical affinities (~2 nM) (table 3). A major difference between hGHR and smGHR lies in their responses to the rat GH. In contrast to the behavior of the hGHR, the smGHR-ECD shows clear binding to the rat GH. The binding affinity of smGHR-ECD to the rat hormone is about 20 times weaker when compared with the interaction between smGHR-ECD and smGH because of the significantly decreased association rate constant value for the interspecies binding interaction (table 3).

GHR-ECD Residue 43 is a Major Determinant of Species Specificity

As described above, the wild-type hGHR-ECD shows no binding affinity toward the rat GH in accord with the well-established species specificity of Old World primate GHRs. However, introduction of a single mutation Arg43Leu provided binding toward the rat hormone that was only slightly weaker than the wild-type hGHR-ECD:hGH interaction (fig. 3A and table 3), whereas it conserved strong binding to the hGH and the smGH. Moreover, we introduced a converse mutation to smGHR-ECD. The resulting variant smGHR-ECD43Arg (with a human residue at position 43) completely lost its affinity toward rat GH (fig. 3B and table 3), while maintaining its affinity toward hGH and smGH. In other words, it acquired a species specificity to respond only to primate GHs. This strongly suggests that the amino acid at position 43 is likely to be the primary determinant for the so-called species specificity: Arg confers, whereas Leu disables, the species specificity.
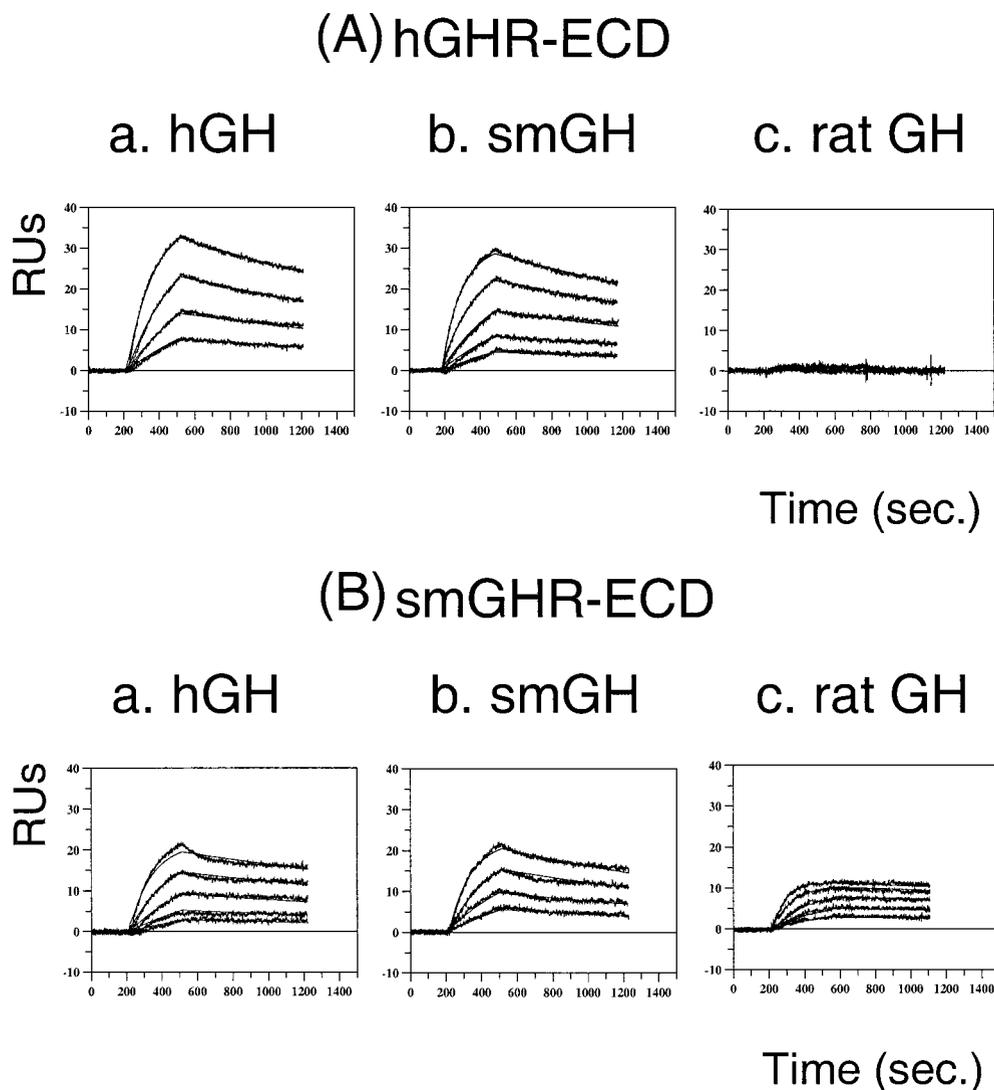
(A) hGHR-ECD

a. hGH

b. smGH

c. rat GH

Time (sec.)

(B) smGHR-ECD

a. hGH

b. smGH

c. rat GH

Time (sec.)

Fig. 2.—(*A*) Representative sensorgrams from hGHR-ECD on the C1 biosensor chip. The simulated sensorgrams from simultaneous and global fit of the experimental data are shown superimposed upon the sensorgrams. (a) Sensorgrams from injections of serial dilutions of hGH in 40-, 20-, 10-, and 5-nM concentrations. (b) Injections of smGH in 80-, 40-, 20-, 10-, and 5-nM concentrations. (c) Injections of serial dilutions of the rat GH as high as 100-nM concentrations. (*B*) Representative sensorgrams from smGHR-ECD on the C1 biosensor chip. (a) Sensorgrams from injections of serial dilutions of hGH in 50-, 25-, 12.5-, and 6.25-nM concentrations. (b) Injections of smGH in 50-, 25-, 12.5-, and 6.25-nM concentrations. (c) Injections of serial dilutions of the rat GH in 100-, 50-, 25-, 12.5-, and 6.125-nM concentrations.

The Effects of Reciprocal Mutations at Residue 43 of the Primate GHR-ECDs

We were able to make comparisons of the binding affinities of interaction between primate GHs and their receptors with regard to position 43 to elucidate the role of residue 43 of the GHR in within-primate interactions (table 3). The hGH binds tightly to both human and squirrel monkey GHR-ECDs. The interaction between hGH and smGHR-ECD was the strongest among the comparisons. Interestingly, the interactions between hGH and hGHR-ECD43Leu and smGHR-ECD43Arg were kinetically very similar (3.9 nM for both cases; table 3).

The purified smGH binds tightly to both its own receptor and hGHR, with 2.3- and 2.0-nM dissociation constants, respectively (table 3). The binding between smGH and smGHR-ECD with the human residue Arg at position 43 was weaker than the wild-type interactions (5.7- nM compared with 2.3-nM dissociation constant). Binding of smGH toward the mutant hGHR-ECD, encoding residue Leu at positon 43, is about two-fold weaker than the binding toward the wild-type hGHR-ECD (6.0- vs. 3.3-nM dissociation constants).

Overall, there is no directional effect of residue 43 on the within-species binding affinities observed. Therefore, in contrast to the dramatic effect of residue 43 on the binding toward the rat GH, within human and squirrel monkey GHRs, whether residue 43 is Leu or Arg does not affect the binding affinities in the same direction. For both species, the binding affinities were greater for the wild-type interactions. At the same time the wild-type interactions were not the strongest; we observed

Table 3
Kinetic Parameters for the Binding of Different GHR-ECDs to hGH, smGH, and Rat GH. Each Experimental Measurement is Repeated at Least Four Times (see Text), and the Average is Shown in the Table

Hormone (number of measurements)	$k_{on} \times 10^5 (M^{-1} s^{-1})$ (SE)[a]	$k_{off} \times 10^5 (s^{-1})$ (SE)[a]	K_d (nM) (SE)[a]	Binding Affinity Relative to WT[b]
hGHR-ECD on the chip				
hGH (5)	1.38 (0.33)	4.83 (1.35)	3.51 (0.46)	1
smGH (2) . . .	1.30 (0.30)	4.23 (0.21)	3.33 (0.60)	1.05
rat GH (4). . .	No binding	No binding	—	No binding
smGHR-ECD on the chip				
hGH (3)	2.27 (0.69)	4.30 (0.97)	1.96 (0.41)	1.17
smGH (3) . . .	1.78 (0.37)	4.08 (0.87)	2.30 (0.13)	1
rat GH (6). . .	0.13 (0.03)	6.13 (4.6)	45.7 (15.8)	0.05
hGHR-ECD Arg43Leu on the chip				
hGH (4)	1.75 (0.74)	6.15 (0.85)	3.93 (1.48)	0.89
smGH (3) . . .	1.23 (0.14)	7.23 (1.54)	6.02 (1.70)	0.58
rat GH (4). . .	1.14 (0.65)	4.86 (2.74)	5.09 (1.67)	0.68
smGHR-ECD Leu43Arg on the chip				
hGH (5)	1.66 (0.43)	6.47 (2.56)	3.89 (1.40)	0.59
smGH (4) . . .	0.94 (0.08)	5.22 (0.82)	5.65 (1.36)	0.46
rat GH (4). . .	No binding	No binding	—	No binding

[a] The standard error (SE) for each measure is shown in parentheses.
[b] Binding affinity relative to WT is in comparison with the interactions between wild-type GHR-ECD and the corresponding GH, i.e., between hGHR-ECD and hGH interaction for the cases involving hGHR-ECD and hGHR-ECD43Leu, and between smGHR-ECD and smGH interaction for the cases involving smGHR-ECD and smGHR-ECD43Arg.

the strongest binding interactions between hGH and smGHR-ECD. Although hGH showed similar binding affinity across the various GHR-ECDs used, smGH showed more variation in terms of binding affinity toward different GHR-ECDs.

Discussion
Effects of Mutations on the Binding Kinetics and Difficulties of Deciphering Protein Evolution from Simple Binding Data

Except for the rat GH:smGHR-ECD interaction, the observed association rates for all the binding assays conducted in this study were similar (table 3). In the case of rat GH:smGHR-ECD, it was the association rate that was more than an order of magnitude lower than those from other types of interactions assessed. The cause for this is unknown; without the knowledge of the three-dimensional structure of the rat GH and the smGHR-ECD, we cannot infer the conformational changes involved in the complex formation. The low association rate also lowered the resolution of the measurements, resulting in a large random error for this specific interaction. In contrast to this, rat GH binds to hGHR-ECD43Leu with an association rate similar to that of primate GHs. Interestingly, the dissociation rate constant values were similarly low for all the GH:GHR-ECD complexes studied. The fact that both hGH and smGH tend to bind weaker to mutant GHR-ECDs suggests that the mutation at residue 43 slightly disturbs the wild-type GHR-ECD structure. In other words, the smGHR-ECD43Arg is a close, but not perfect, substitute for hGHR-ECD, and the same is true for the other mutant GH–wild-type GHR pair. Other nearby differences of

residue conformations in the three-dimensional structures might cause such an effect.

Inferring the selective advantage or disadvantage of a specific amino acid substitution by means of functional data is not straightforward. For example, the effects of mutations may not be additive, but synergistic. Indeed, Clackson et al. (1998) reported that some of the mutations outside the structural epitopes of hGHR could indirectly influence the binding affinity. Therefore, inferring the significance of individual residues serves as only the first step in understanding the phenotypic consequences of specific substitutions. Another limitation of the measurement of choice presented in this report lies in the fact that the present study focused only on the interaction between GHR-ECDs and GHs through site 1 binding. It should be noted that the species specificity was originally described in a biological system (Carr and Friesen 1976) in which both site 1 and site 2 interactions occur. The site 1 binding affinity accounts for the first step of the cascade of the biological signal transduction pathway (Kossiakoff 1995). It is possible that the kinetics of site 1 and site 2 binding may behave differently in response to a single mutation. Unfortunately, this aspect of GH:GHR interactions has not been investigated in detail yet because of the fact that it is technically challenging to measure the site 1 and site 2 binding affinities separately. A new SPR technique for this purpose, however, has been developed recently (Bernat et al., unpublished data). Determining whether the effect of a mutation that is critical in site 1 binding affinity, such as the GH171 or GHR43 residue, has the same degree of significance at the site 2 binding affinity will be important to elucidate the underlying biological
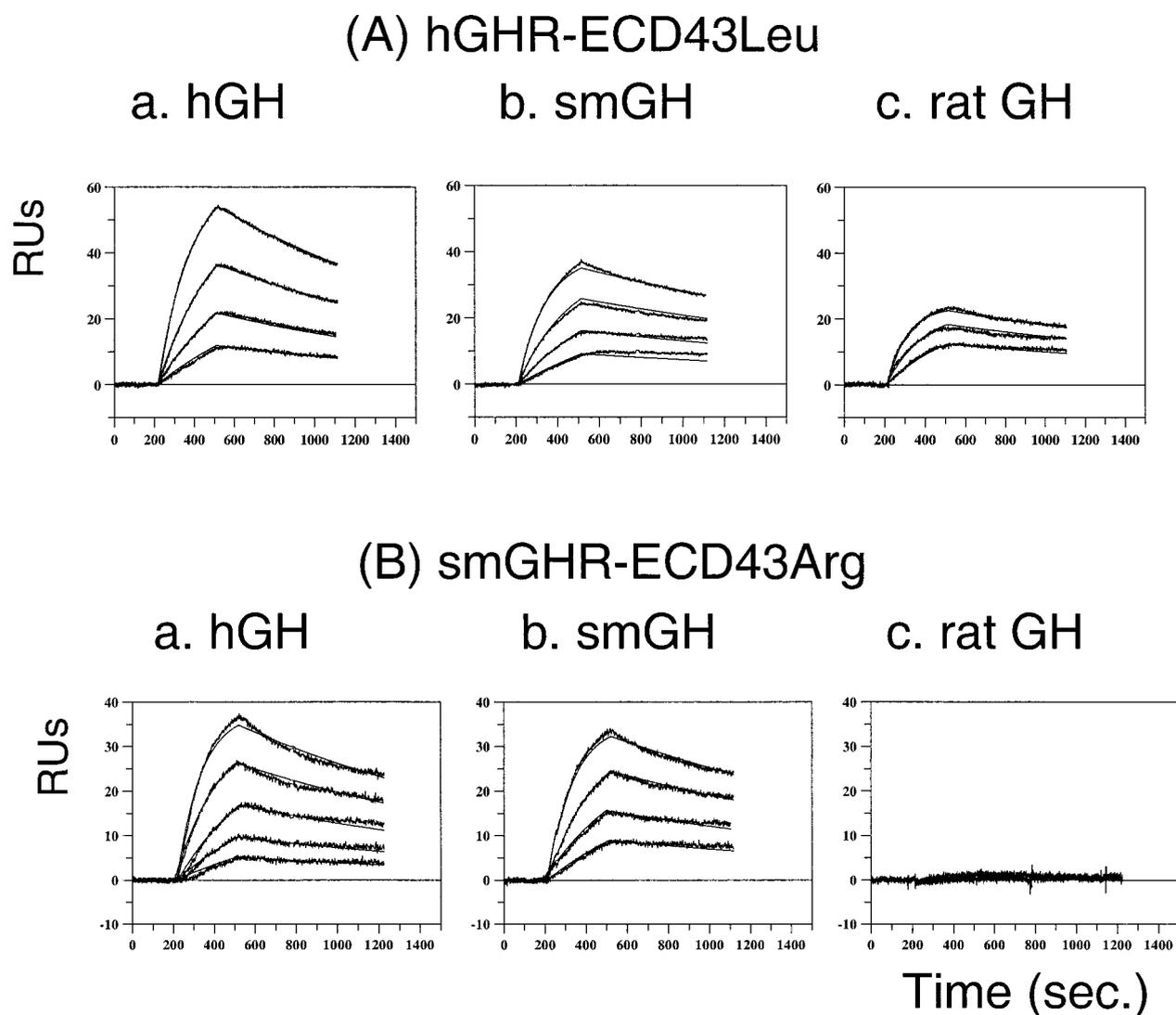
(A) hGHR-ECD43Leu

a. hGH   b. smGH   c. rat GH

(B) smGHR-ECD43Arg

a. hGH   b. smGH   c. rat GH

Time (sec.)

FIG. 3.—(*A*) Representative sensorgrams from hGHR-ECD43Leu on the C1 biosensor chip. (a) Sensorgrams from injections of serial dilutions of hGH in 40-, 20-, 10-, and 5-nM concentrations. (b) Injections of smGH in 80-, 40-, 20-, 10-, and 5-nM concentrations. (c) Injections of serial dilutions of the rat GH in 100-, 50-, and 25-nM concentrations. (*B*) Representative sensorgrams from smGHR-ECD43Arg on the C1 biosensor chip. (a) Sensorgrams from injections of serial dilutions of hGH in 50-, 25-, 12.5-, 6.25-, and 3.125-nM concentrations. (b) Injections of smGH in 80-, 40-, 20-, 10-, and 5-nM concentrations. (c) Injections of serial dilutions of the rat GH in as high as 100-nM concentrations.

consequences and to facilitate the understanding of the trajectories of functional evolution.

Origin and the Evolution of the Major Determinant of Old World Primate GHR Specificity

The species specificity of the Old World primate GHR is an example of the dramatic functional differences of the same proteins from different phylogenetic units. Although most of the hormones produced in one mammalian species are physiologically effective when injected into another species, the incompatibility of human and Rhesus monkey GHRs toward nonprimate GHs stands out as a notable exception (Carr and Friesen 1976).

Efforts to elucidate the molecular basis of species specificity became successful only after detailed structural and biochemical knowledge of the GH and GHR interaction became available. A high-resolution X-ray structure of the ternary hGH·(hGHR-ECD)$_2$ complex (De Vos, Ultsch, and Kossiakoff 1992) revealed the amino acid residues that are directly involved in the formation of the ternary complex (table 1). Complementary binding studies using systematic mutagenesis strategies both on the hormone (Cunningham and Wells 1989; Cunningham et al. 1989) and on the receptor (Clackson and Wells 1995; Clackson et al. 1998) identified the most important residues for the site 1 interaction (table 1). On the basis of this information, it was speculated that the structurally complementary residues Asp171 of the hormone and Arg43 of the receptor were the principal determinants of species specificity (Souza et al. 1995).

Recently, Liu et al. (2001) and Wallis, Zhang, and Wallis (2001) studied three New World monkey species, i.e., squirrel monkey, spider monkey, and marmoset, and

found that position 171 of the GH is an Asp in all three species, suggesting that the His → Asp change occurred before the radiation of New World monkeys. Liu et al. (2001) further found that the GHR of the squirrel monkey is encoded by Leu at position 43, the so-called nonprimate residue. Therefore, the states of the GH and the GHR in the New World monkeys would appear to be the "missing link" of the emergence of species specificity. Whether the situation of the squirrel monkey really represents a functional intermediary state was the motivating question of this study. Our functional analysis supported this hypothesis. Thus, the species specificity is an Old World primate–specific trait; smGHR can bind to the nonprimate GH as well as to the primate GH, in contrast to the hGHR, which can only bind to the higher primate GH. We further demonstrated that this functional characteristic could be altered by a single amino acid change at residue 43. In other words, we were able to reconstruct the evolutionary trajectory of the emergence of species specificity; it can occur through a single nucleotide change (CTA → CGA) in New World monkey GHR, where the GH171His → GH171Asp substitution has already occurred (Liu et al. 2001).

Biochemical Basis of the Major Determinant of Old World Primate GHR Specificity

There are stereochemical reasons why the His → Asp mutation in the hormone must have preceded the Leu → Arg mutation in the receptor. The GHR43Arg residue is known to be the center of an extensive network of structural interactions necessary for the stable hormone-receptor complex (De Vos, Ultsch, and Kossiakoff 1992; Behncken et al. 1997; Clackson et al. 1998). The hGH:hGHR interaction is stabilized by a salt bridge between hGHR43Arg and hGHAsp171 and by a hydrogen bond between hGH175Thr and hGHR43Arg (fig. 1). In addition, the alkyl portion of the side chain of Arg43 of the hGHR is known to form extensive van der Waals interactions with the side chain of Trp169 and also contacts Ile103 and Trp104 (fig. 1). Because of the extensive array of interactions involving GHR43Arg, it is to be expected that any change in this residue will result in a substantial decrease in affinity.

However, when changed to Leu, we did not observe a significant reduction in site 1 binding affinity (table 3; see also Clackson et al. 1998). This leads to a suggestion that the Arg43 guanidium group interactions are not as important as the hydrophobic interaction with the receptor Trp169, which presumably still occurs with Leu residue at GHR43 (Behncken et al. 1997; Clackson et al. 1998). Leu can maintain this intramolecular packing, even though it lacks the electrostatic charge caused by the deletion of the guanidinium group (see fig. 3 of Behncken et al. 1997 for suggested structural interface for this interaction).

Substitution of Asp by His at GH171 will cause a steric hindrance and repulsive interactions with Arg43 (see fig. 3 of Behncken et al. 1997) and, therefore, result in the observed incompatibility. However, with

GHR43Leu this hindrance is removed; hence, no change in site 1 affinity is observed. Therefore, although the GHR43 change could not have preceded the GH171 change (because it will lose affinity to its own GH), once the GH171 change occurred, the Leu → Arg substitution became possible without a dramatic change in the binding affinity.

Was the Emergence of the Species Specificity of GHR the Result of Positive Selection?

What triggered the evolution of the GH171Asp: GHR43Arg pair as seen in Old World primates? A selective hypothesis postulates that these two residues co-evolved toward increased affinity and specificity; in other words, the species specificity evolved as a result of positive selection. From our binding analysis, we found no direct evidence to support this hypothesis. For the smGHR, the pairs of molecules engineered to have the GH171Asp:GHR43Arg interaction exhibited a lower binding affinity than the wild-type interaction, which is intermediary in the evolution of functional specificity (GH171Asp:GHR43Leu). In the case of the hGHR, the intermediary interaction had essentially the same binding affinity as the wild-type interactions (GH171Asp: GHR43Arg). Therefore, although the incompatibility between GH171His and GHR43Arg was the major determinant of the Old World primate GHR specificity, the interaction between GH171Asp and GHR43Arg does not suggest selective advantage, at least starting from the sequence of smGHR. It should also be noted that in these measurements the hGH had a high affinity toward smGHR-ECD, which has Leu at residue 43. Therefore, for the interaction between GH and GHRs in Old World primates, the GH171Asp:GHR43Arg interaction does not appear to be a significant improvement over the intermediate status of GH171Asp:GHR43Leu.

Additionally, Liu et al. (2001) reported that the intermediary state of the GH171Asp:GHR43Leu pair is found in both squirrel monkey and spider monkey. These two species represent the two most divergent families of the Haplorhine Superfamily Ceboidea, which has an estimated age of 25 Myr (Goodman et al. 1998). This indicates that the intermediate state has persisted for a very long period of time within Platyrrhines. If the new mutation of GHR43Leu → GHR43Arg has a significant selective advantage, then the intermediary state is more likely to be short-lived. The observation that there is no increase in binding affinity for the evolved interaction compared with the ancestral interaction and the phylogenetic distribution of this substitution both indicate that the evolution of the GHR species specificity of Old World primates may not be a direct consequence of selectively driven coevolutionary substitutions in the GH and the GHR.

An alternative explanation is that the new mutation (T → G) was selectively neutral and became fixed in the population by chance, and the evolution of the species specificity is a byproduct of this substitution. The lack of direct evidence for a selective advantage of the new mutation in the smGHR favors this explanation. In

the light of this conclusion, it will be of great interest to investigate whether the GH171His → GH171Asp change had any significant selective advantage. This substitution provided the biochemical basis for the emergence of the species specificity of Old World primate GHRs. Interestingly, the timing of this substitution coincides with the acceleration in the evolutionary rate observed in the ancestral lineage leading to higher primates (Liu et al. 2001).

Acknowledgments

LITERATURE CITED

BEHNCKEN, S. N., S. W. ROWLINSON, J. E. ROWLAND, B. L. CONWAY-CAMPBELL, T. A. MONKS, and M. J. WATERS. 1997. Aspartate 171 is the major primate-specific determinant of human growth hormone. J. Biol. Chem. **272**:27077–27083.

CARR, D., and H. G. FRIESEN. 1976. Growth hormone and insulin binding to human liver. J. Clin. Endocrinol. Metab. **42**:484–493.

CLACKSON, T., M. H. ULTSCH, J. A. WELLS, and A. M. DE VOS. 1998. Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity. J. Mol. Biol. **277**:1111–1128.

CLACKSON, T., and J. A. WELLS. 1995. A hot spot of binding energy in a hormone-receptor interface. Science **267**:383–386.

CUNNINGHAM, B. C., P. JHURANI, P. NG, and J. A. WELLS. 1989. Receptor and antibody epitopes in human growth hormone identified by homolog-scanning mutagenesis. Science **243**:1330–1336.

CUNNINGHAM, B. C., and J. A. WELLS. 1989. High-resolution epitope mapping of hGH-receptor interaction by alanine-scanning mutagenesis. Science **244**:1081–1085.

———. 1991. Rational design of receptor-specific variants of human growth hormone. Proc. Natl. Acad. Sci. USA **88**:3407–3411.

———. 1993. Comparison of a structural and a functional epitope. J. Mol. Biol. **234**:554–563.

DE VOS, A. M., M. ULTSCH, and A. KOSSIAKOFF. 1992. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science **255**:306–312.

FISHER, R. J., M. FIVASH, J. CASAS-FINET, J. W. ERICKSON, A. KONDOH, S. V. BLADEN, C. FISHER, D. WATSON, and T. PAPAS. 1994. Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms. Protein Sci. **3**:257–266.

FUH, G., M. G. MULKERRIN, S. BASS, N. MCFARLAND, M. BROCHIER, J. H. BOURELL, D. R. LIGHT, and J. A. WELLS. 1990. The human growth hormone receptor. J. Biol. Chem. **265**:3111–3115.

GOODMAN, M., C. A. PORTER, J. CZELUSNIAK, S. L. PAGE, H. SCHNEIDER, J. SHOSHANI, G. GUNNELL, and C. P. GROVES. 1998. Toward a phylogenetic classification of primates based on DNA evidence complemented by fossil evidence. Mol. Phylogenet. Evol. **9**:585–598.

KOSSIAKOFF, A. 1995. Structure of the growth hormone-receptor complex and mechanism of receptor signaling. J. Nucl. Med. **36**:14S–16S.

KUNKEL, T. A., J. D. ROBERTS, and R. A. ZAKOUR. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.

LI, W.-H. 1997. Molecular evolution. Sinauer Associates, Sunderland, Mass.

LIU, J.-C., K. D. MAKOVA, R. M. ADKINS, S. GIBSON, and W.-H. LI. 2001. Episodic evolution of growth hormone in primates and emergence of the species specificity of human growth hormone receptor. Mol. Biol. Evol. **18**:945–953.

PETERSON, F. C., and C. L. BROOKS. 2000. The species specificity of growth hormone requires the cooperative interaction of two motifs. FEBS Lett. **472**:276–282.

SOMERS, W., M. ULTSCH, A. M. DE VOS, and A. A. KOSSIAKOFF. 1994. The X-ray structure of a growth hormone-prolactin receptor complex. Nature **372**:478–481.

SOUZA, S. C., G. PETER FRICK, X. WANG, J. J. KOPCHICK, R. B. LOBO, and H. M. GOODMAN. 1995. A single arginine residue determines species specificity of the human growth hormone receptor. Proc. Natl. Acad. Sci. USA **92**:959–963.

WALLIS, M. 1994. Variable evolutionary rates in the molecular evolution of mammalian growth hormones. J. Mol. Evol. **38**:619–627.

———. 1996. The molecular evolution of vertebrate growth hormone: a pattern of near-stasis interrupted by sustained bursts of rapid change. J. Mol. Evol. **43**:93–100.

WALLIS, O. C., Y.-P. ZHANG, and M. WALLIS. 2001. Molecular evolution of GH in primates: characterization of the GH genes from slow loris and marmoset defines an episode of rapid evolutionary change. J. Mol. Endocrinol. **26**:249–258.

WELLS, J. A. 1994. Structural and functional basis for hormone binding and receptor oligomerization. Curr. Biol. **6**:163–173.