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The Functional Binding Epitope of a High Affinity Variant of Human Growth Hormone Mapped by Shotgun Alanine-scanning Mutagenesis: Insights into the Mechanisms Responsible for Improved Affinity

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A high-affinity variant of human growth hormone (hGH_v) contains 15 mutations within site 1 and binds to the hGH receptor (hGHR) ~400-fold tighter than does wild-type (wt) hGH (hGH_{wt}). We used shotgun scanning combinatorial mutagenesis to dissect the energetic contributions of individual residues within the hGH_v binding epitope and placed them in context with previously determined structural information. In all, the effects of alanine substitutions were determined for 35 hGH_v residues that are directly contained in or closely border the binding interface. We found that the distribution of binding energy in the functional epitope of hGH_v differs significantly from that of hGH_{wt}. The residues that contributed the majority of the binding energy in the wt interaction (the so-called binding "hot spot") remain important, but their contributions are attenuated in the hGH_v interaction, and additional binding energy is acquired from residues on the periphery of the original hotspot. Many interactions that inhibited the binding of hGH_{wt} are replaced by interactions that make positive contributions to the binding of hGH_v. These changes produce an expanded and diffused hot spot in which improved affinity results from numerous small contributions distributed broadly over the interface. The mutagenesis results are consistent with previous structural studies, which revealed widespread structural differences between the wt and variant hormone–receptor interfaces. Thus, it appears that the improved binding affinity of hGH_v site 1 was not achieved through minor adjustments to the wt interface, but rather, results from a wholesale reconfiguration of many of the original binding elements.

Keywords: phage display; alanine-scanning; shotgun scanning; protein–protein interactions; combinatorial mutagenesis

Introduction

The initiating event in many signal transduction pathways is the specific binding of a hormone to the extracellular domain of a transmembrane receptor. These extracellular binding interactions can oligomerize receptors and, in turn, this can lead to the activation of intracellular signaling molecules such as kinases and transcription factors.[1–3] Because hormone–receptor interactions are central regulators of biology, a detailed understanding of how these protein–protein interactions evolve and function is of great fundamental and practical importance.

The interactions between human growth hormone (hGH) and its receptors represent perhaps the best characterized protein–protein interactions.[1,4–10] Both X-ray crystallography and biochemical methods have been used to show that hGH sequentially binds two identical receptors to form an active 2:1 signaling complex.[5,11] In the first

Abbreviations used: BSA, bovine serum albumin; ECD, extracellular domain; ELISA, enzyme-linked immunosorbant assay; hGH, human growth hormone; hGH_v, high-affinity hGH variant; hGH_{wt}, wild-type hGH; hGHR, human growth hormone receptor; hPRLR, human prolactin receptor; PRL, prolactin; wt, wild-type.

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step, hGH binds to a single receptor molecule through a high affinity binding site (site 1), and subsequently, recruits a second copy of the receptor through a lower affinity secondary binding site (site 2). The site 1 interaction has been subjected to extensive site-directed and combinatorial mutagenesis to elucidate the requirements for binding affinity and specificity, and to study how protein–protein interactions evolve.[4–7,12–15]

The biological functions of GH are integrated with those of the hormone prolactin (PRL), and they are governed by a sophisticated mixture of receptor specificity and cross-reactivity.[16–18] For example, hGH can bind both to the hGH receptor (hGHR) and to the human PRL receptor (hPRLR), while in contrast, hPRL binds only to hPRLR. The hormones and receptors of the GH/PRL system presumably evolved from common hormone and receptor gene parents,[11,16–19] and their distinct functions were refined during the 400 million years since they diverged. The specificity and cross-reactivity traits of the GH/PRL system involve significant molecular recognition challenges, since both the hormones and receptors have little sequence conservation (~25%) even among the residues involved in the hormone–receptor binding interfaces.[20]

The nature of the structural diversity operating in these systems, accounting for both the extensive changes required to recognize significantly different binding sites and those producing the subtle effects that generate specificity, is a topic of intense investigation.[21] It is a question of broad biological importance whether the complexity of such versatile binding features depends on a narrowly evolved set of solutions, or on the contrary, whether many extremely different solutions can provide the same binding capabilities. If other binding solutions exist, how different are they and what are their structural and biochemical characteristics?

Some of these issues have been addressed by a series of molecular evolution experiments.[12–15,22] Using phage display methods, Wells and colleagues dramatically increased the affinity of hGH site 1 binding to the hGHR.[14,22] Mutations that provided modest increases in affinity were first selected and then combined to eventually produce an extremely high affinity hGH variant (hGH_v) which contained a total of 15 mutations and bound to the hGHR ~400-fold more tightly than did wild-type hGH (hGH_{wt}). As an additional consequence of the affinity maturation process, hGH_v lost the ability to bind to hPRLR.

The recent structure determination of hGH_v bound to two copies of the hGHR extracellular domain (ECD) revealed that significant reorganization occurs at the site 1 interface.[23] In fact, very few H-bonding interactions are conserved even between residues that were not mutated. This demonstrated that improved affinity was evolved not only through the introduction of novel interactions mediated directly by mutated residues, but also by conformational changes in non-mutated regions of the interface. Thus, the repertoire of diversity explored by the selection process was not limited to sequence changes at mutagenized sites, but instead, was greatly expanded by structural plasticity effects extending across the entire interface.

Because the binding of hGH_v is governed by a quite different set of structure–function relationships in comparison to that of hGH_{wt}, we felt it would be of significant general interest to dissect the energetic components of the affinity matured site 1 interface. Determination of the energetic contributions of individual residues in the high affinity interface, however, posed significant experimental difficulties. The number of mutations that we wished to explore was large and included residues not only in the interface, but also a set of bystander groups that could potentially contribute to binding through indirect, long-range effects. Furthermore, the binding of the variant was so tight that the standard biochemical approaches used for the analysis of hGH_{wt} were not feasible (G.P. and A.A.K., unpublished results).

To circumvent the problems inherent in determining accurately the affinities of very tight binding associations, we used a recently developed approach called shotgun alanine-scanning.[24–26] This method combines alanine-scanning and phage display mutagenesis in a binomial format; phage-displayed protein library members vary as wild-type (wt) or Ala with equal frequency at the targeted sites. The method is extremely rapid because many side-chains can be analyzed simultaneously with no need for protein purification or direct biophysical analysis. Instead, the energetic contributions of the individual side-chains are derived from a statistical analysis of the DNA sequences of a large number of unique library members. It has been demonstrated that the determined wt/Ala ratios at individual positions can be used to predict the effects of individual Ala substitutions on binding affinity, and these predictions are in good agreement with results obtained by classical alanine-scanning procedures.[24]

We report here the determination of the binding energetics for 35 hGH_v site 1 residues using shotgun alanine-scanning. We present a functional map that describes the energetic contributions for each residue in the hGH_v site 1 interface. Together with previous structural data and alanine-scanning data for hGH_{wt}, the results offer a uniquely comprehensive view of the molecular mechanisms through which multiple mutations can alter and enhance protein–protein binding interactions.

Results and Discussion

The structure of the hGH_v–hGHR complex

The high affinity hGH_v variant contains 15 mutations within the site 1 hormone–receptor

binding interface. Although, as a group, these mutations greatly increased affinity, many of these substitutions were non-homologous to the residue types they replaced.[22] Clearly, the process of affinity maturation was not one of subtle tuning of existing properties. For instance, in a number of cases, groups that made H-bonds or salt-bridges to receptor residues were substituted with hydrophobic residues. Additionally, the X-ray structure of the free form of hGH_v showed substantial structural differences compared to its wt counterpart.[27] It is noteworthy that attempts to model the site 1 contacts to the receptor based on the structure of free hGH_v and the model of the wt complex were unsuccessful.[27] This is because the global and local structural changes that were required for both hGH_v and the receptor to produce any reasonable contact interface stereochemistry were too large to be modeled with any confidence.

Recently, the X-ray crystal structure of hGH_v bound to two copies of the hGHR ECD has been determined.[23] The affinity matured hGH_v binds two molecules of the hGHR ECD in a manner similar to that observed in the wt complex. However, as expected based on the non-homologous nature of some of the mutations, there are considerable differences in the details of the specific interactions at the hormone–receptor interfaces. Interestingly, of the ten intermolecular H-bonds between hGH_v and the receptor in site 1 (hGHR1), only one is conserved amongst the eight found in the wt site 1 interaction. Additionally, the contact surface at hGH_v site 1 (1180 Å^2) is somewhat smaller than that found in the wt interaction (1300 Å^2). As predicted from the modeling attempts, there are substantial conformational changes between the free and bound forms of hGH_v, some as large as 9 Å.[23]

Because of the extent of global and local changes to both the hormone and the receptor, it was not possible to assign a structural rationale for which groups and contacts were most responsible for the improved affinity of hGH_v site 1. Therefore, to gain insights into the sequence and conformational changes that contribute to the improved affinity of hGH_v, we undertook a comprehensive evaluation of the energetic contributions of all the residues in the site 1 interface of the hormone. The results are presented and discussed below.

Shotgun alanine-scan of hGH_v

Shotgun alanine-scanning uses phage-displayed libraries to analyze multiple sites in a protein simultaneously. To do this, special "shotgun alanine-scan" codons are used to vary each site as only the wt or Ala, although the redundancy of the genetic code necessitates two additional amino acid substitutions for some residues.[24] We constructed a shotgun alanine-scanning library covering 35 residues that constitute essentially the entire structural epitope for site 1 binding of hGH_v to the hGHR.[23] Because the structural epitope is discontinuous, library construction required the use of four mutagenic oligonucleotides designed to introduce mutations in four distinct regions of secondary structure: helix-1 (residues 14, 18, 21, 22, 25, 26, and 29), mini-helix (residues 41–48), loop-60 (residues 60–68), and helix-4 (residues 164, 167, 168, 171, 172, 174, 175, 176, 178, 179, and 183).

The library covered 13 of the 15 residues that differ in hGH_v compared with hGH_{wt}; residue 10 was not included because it is an Ala in hGH_v. Pro54 also could not be included because it lies between the mini-helix and loop-60, and the library construction methods do not allow for mutations in regions that would require sequence overlap between independent mutagenic oligonucleotides.[28]

The library was subjected to two separate selections. In the first selection, the hGHR ECD was used as the capture target and was the basis for determining wt/Ala ratios used to calculate the changes in the binding free energies for the Ala substitutions. In the second selection, library phage were captured with a monoclonal antibody that binds to hGH in a region that does not overlap the mutated region.[29] The antibody selections were used to identify and quantify decreases in the level of hGH displayed on phage, either through expression effects or structural perturbations that lead to misfolded proteins that are degraded through proteolysis.

Approximately 100 or 200 binding clones were sequenced from the antibody selection or the hGHR ECD selection, respectively. For each selection, the sequences were aligned and used to calculate the wt/Ala ratio at each mutated position (see Materials and Methods). The wt/Ala ratio correlates with the effect of each mutation on the selected trait,[24] with ratios greater than or less than 1 indicating deleterious or beneficial mutations, respectively (Table 1). The wt/Ala ratios for the antibody selection were close to 1 for most of the positions. This was an expected result, since the side-chains we investigated are almost all surface exposed, and thus, unlikely to effect protein stability. It is noteworthy, however, that three of the residues that differ in hGH_v compared with hGH_{wt} (Trp14, Asn21, and Asn167) exhibited wt/Ala ratios much less than 1, suggesting that, while these mutations were selected for improved binding to the hGHR, they may actually compromise the structural stability of hGH_v and lead to reduced levels of display on phage.

Calculation of $\Delta\Delta G$ from the phage selections

For each of the selections, the wt/Ala ratios at each mutated position were used to calculate "statistical $\Delta\Delta G$ values" that measured the effects of individual mutations on the selection. To correct for effects on levels of hGH_v display, the $\Delta\Delta G$ values for the antibody selection were subtracted from those for the hGHR ECD selection. This

Table 1. Shotgun scanning analysis of hGH_v

	wt/mutant ratios								
	hGHR selection			Antibody selection			$\Delta\Delta G_{mut-wt}$		
Residue[a]	wt/Ala	wt/m2	wt/m3	wt/Ala	wt/m2	wt/m3	Ala	m2	m3
W14M	3.03	8.33	2.50	0.13	0.46	0.22	**1.9**	1.7 (G)	1.4 (S)
D18H	1.14			0.42			**0.6**		
N21H	0.19	2.1	0.82	0.11	7.00	1.02	0.3	−0.6 (D)	−0.1 (T)
Q22	0.71	1.74	16.5	0.50	0.79	ND[b]	0.2	0.6 (E)	ND[b](P)
F25	1.79	1.73	2.30	1.00	2.30	1.67	0.3	−0.2 (S)	0.2 (V)
D26	0.45			0.84			−0.4		
Q29	0.83	3.23	17.8	0.49	1.41	ND[b]	0.3	0.5 (E)	ND[b] (P)
I41K	0.44	1.09	0.32	0.50	1.40	0.33	−0.1	−0.1 (E)	0.0 (T)
H42Y	0.6	1.42	1.26	0.74	1.47	1.39	−0.1	0.0 (D)	0.0 (P)
S43	1.19			0.94			0.1		
F44	1.52	1.79	1.30	1.52	1.78	1.62	0.0	0.0 (S)	−0.1 (V)
W45L	2.58	2.14	1.67	0.78	0.72	0.67	**0.7**	0.7 (G)	0.1 (S)
W46Q	2.25	1.19	1.62	0.81	0.41	1.21	**0.6**	0.6 (G)	0.2 (S)
N47	0.31	1.15	1.00	0.46	0.94	0.85	−0.2	0.1 (D)	0.1 (T)
P48	1.75			2.17			−0.1		
T60	1.27			0.94			0.2		
P61	2.94			0.63			**0.9**		
S62	1.16			1.44			−0.1		
N63	2.29	3.56	0.86	0.76	1.22	0.95	**0.7**	0.6 (D)	−0.1 (T)
K64R	6.65	51.0	30.6	0.77	1.00	1.35	**1.3**	2.3 (E)	1.9 (T)
E65	0.41			1.56			−0.8		
E66	1.2			0.74			0.3		
T67	1.27			0.42			**0.7**		
Q68	0.93	3.45	19.0	0.97	1.65	1.65	0.0	0.4 (E)	1.4 (P)
Y164	1.55	9.9	1.80	1.16	18.5	1.76	0.2	−0.4 (D)	0.0 (S)
N167R	0.25	4.5	0.73	0.095	7.02	0.78	**0.6**	−0.3 (D)	−0.0 (T)
K168	1.18	3.53	1.81	1.60	9.05	3.20	−0.2	−0.6 (E)	−0.3 (T)
S171D	2.98			0.89			**0.7**		
K172	7.89	15.8	14.2	0.25	0.69	0.61	**2.0**	1.9 (E)	1.9 (T)
S174E	1.31			0.92			0.2		
T175	2.73			0.64			**0.9**		
Y176F	9.63	77.0	22.0	1.03	6.03	1.11	**1.3**	1.5 (D)	1.8 (S)
R178	11.1	31.0	31.0	2.88	1.77	ND[b]	**0.8**	1.7(G)	ND[b] (P)
T179I	1.02			0.68			0.2		
R183	4.4	10.2	66.0	0.32	2.50	20.0	**1.6**	0.8 (G)	0.7 (P)

For each of the 35 scanned positions, the wt/mutant ratios were determined from the sequences of clones isolated after selection for binding to either the hGHR or an anti-hGH monoclonal antibody. In addition to the effects of Ala substitutions, the effects of two other mutations were also determined in cases where tetranomial codons were used (m2 and m3). These data were used to calculate the difference in binding free energy due to each mutation ($\Delta\Delta G_{mut-wt}$), as described in Materials and Methods. Residues in bold have $\Delta\Delta G_{Ala-wt} > 0.6$ kcal/mol. The identities of the mutations m2 and m3 depend on the particular shotgun codon used and are shown in parentheses to the right of each $\Delta\Delta G_{mut-wt}$ value.

[a] The hGH_v residue is denoted by the single letter amino acid code, followed by a number denoting its position in the hGH sequence, and in positions where the sequences of hGH_{wt} and hGH_v differ, the corresponding residue in hGH_{wt} (e.g. W14M denotes position 14 which is Trp or Met in hGH_v or hGH_{wt}, respectively).

[b] ND indicates that these values were not determined, because these mutations were not observed amongst the antibody selection sequences.

normalization step resulted in an estimate of $\Delta\Delta G_{Ala-wt}$, the difference in binding free energy between alanine-substituted and the native variant (hGH_v) for binding to the hGHR ECD (Table 1).

Because of the degeneracy of the codons used in the shotgun scan, there are a number of residues that are represented by two other amino acid possibilities besides wt and Ala. For instance, in the case of Asn, the additional two amino acid types are Asp and Thr, which are reasonably stereochemically similar. In contrast, for Trp the two additional amino acid types are Gly and Ser, which are stereochemically quite different from the wt amino acid. Nevertheless, these additional substitutions can add to the information content of the scan.[26] The data can be used to calculate $\Delta\Delta G$ values for these substitutions in a manner analogous to that for the Ala substitutions (see columns m2 and m3 in Table 1), although the reliability of these additional predictions remains to be demonstrated for the hGH system.

The functional epitope of hGH_v site 1 binding to the hGHR

Figure 1A shows the $\Delta\Delta G_{Ala-wt}$ values of the 35 alanine-scanned residues mapped onto the site 1 surface, which is characterized by a more dispersed distribution of energy than was previously observed for the hGH_{wt} site 1 interaction (Figure 1B).[6] Ala substitutions for 15 residues, representing ~40% of the interface, produced effects greater than 0.6 kcal/mol (bold text in Table 1). It is noteworthy that the sum of the reductions in

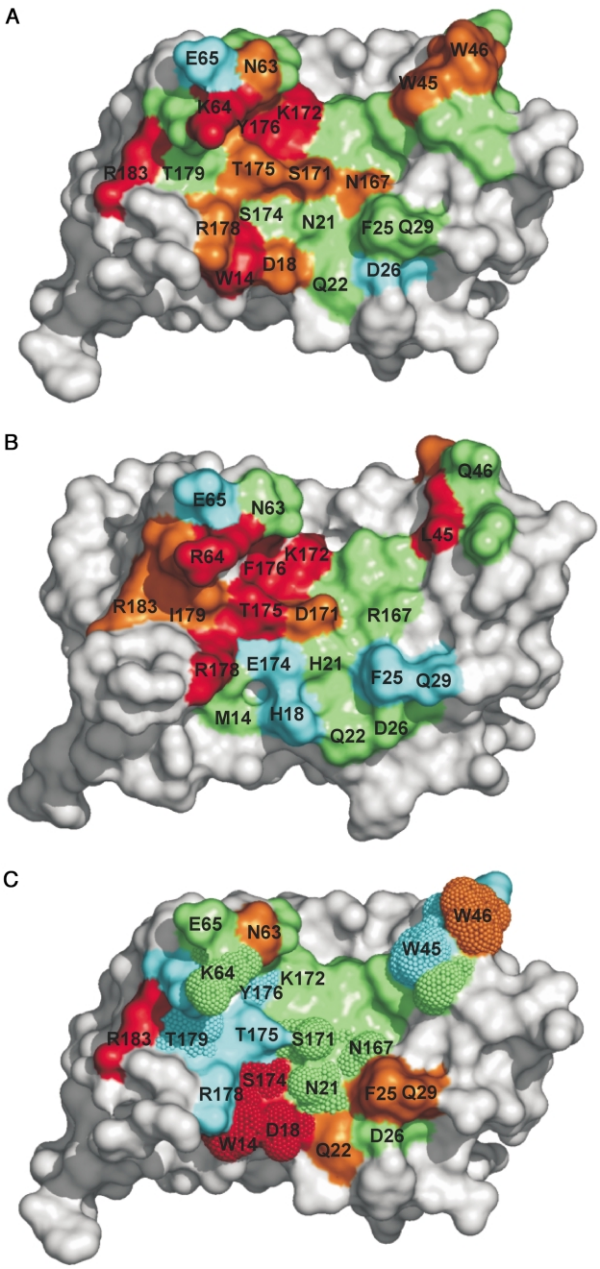

Figure 1. Comparison of the site 1 functional epitope of hGH_v to that of hGH_{wt} for binding to the hGHR. A, The functional epitope of hGH_v defined by shotgun alanine-scanning mapped onto the structure derived from the 2:1 $hGHR/hGH_v$ structure.[23] B, the functional epitope of hGH_{wt} defined by conventional alanine-scanning mapped onto the structure derived from the 2:1 $hGHR/hGH_{wt}$ structure.[11] C, The binding energy difference, obtained by subtracting $\Delta\Delta G_{Ala-wt}$ for hGH_{wt} from $\Delta\Delta G_{Ala-wt}$ for hGH_v mapped onto the structure of hGH_v. Residues in hGH_v that are mutations relative to hGH_{wt} are shown with "bumpy" surfaces. The residues are color coded as follows: red, >1.0 kcal/mol; orange, 0.4 to 1.0 kcal/mol; green, 0.4 to −0.4 kcal/mol; blue, <−0.4 kcal/mol; and gray; untested. Data for hGH_v are from Table 1, while data for hGH_{wt} are from Cunningham and Wells.[6] Structures were rendered in Pymol (DeLano Scientific, San Carlos, CA).

free energies caused by the Ala substitutions (−15.6 kcal/mol) is close to the total free energy of binding between hGH_v and the hGHR ECD (−16.4 kcal/mol) measured by radioimmune assay.[22] Similar good agreement was previously reported for the hGH_{wt}–hGHR site 1 interaction. As noted by Cunningham and Wells,[6] good agreement between the sum of individual mutational effects and the total binding free energy suggests that the effects caused by the Ala substitutions are localized and do not perturb the global protein structure or mechanism of binding.

Of the 15 side-chains that contribute substantial binding energy, 13 are spatially clustered on one side of the hGH_v surface. These residues form an extended patch that contributes a substantial portion of the binding energy (14.0 kcal/mol). This patch is further buttressed by several residues (Asn21, Phe25 and Gln29) that make smaller, but still significant, contributions to binding, and thus further increase the contiguous area of favorable binding interactions. A much smaller satellite of binding energy consists of Trp45 and Trp46, which are located in the mini-helix.

Overall, the structural epitope of hGH_v site 1 is well adapted for binding to the hGHR, since most of the contact surface makes at least somewhat favorable contributions to the binding energy and much of the remaining surface is energetically neutral. The only two significantly negative interactions are located at either end of the structural contact surface (Asp26 and Glu65), and it seems possible that the affinity of hGH_v may be further improved by mutations at these positions.

Comparison of the site 1 functional epitope of hGH_v to that of hGH_{wt}

The interaction between hGH_{wt} site 1 and the hGHR was the first large protein–protein binding interface subjected to a systematic alanine-scan, and these studies led to the binding "hot spot" paradigm.[4,6,7,12] In that analysis, the effects of individual Ala substitutions indicated that a few spatially clustered residues, representing only about one-quarter of the structural binding epitope, accounted for over 85% of the binding energy. Seven of these residues (Leu45, Pro61, Arg64, Lys172, Thr175, Phe176 and Arg178) contributed over 1.0 kcal/mol each and two others (Asp171 and Ile179) contributed over 0.8 kcal/mol each (Figure 1B). Another notable feature of the hGH_{wt} site 1 interaction was that more than half of the structural binding epitope residues were found to be either neutral or inhibitory for binding to the hGHR, as Ala substitutions at these positions either did not affect or even improved the binding affinity.[6]

Figure 2 graphs the $\Delta\Delta G_{Ala-wt}$ values for the 35 hGH_v residues that were analyzed in the present study and compares them with those previously determined for hGH_{wt} with conventional alanine-scanning mutagenesis.[6] The high affinity variant

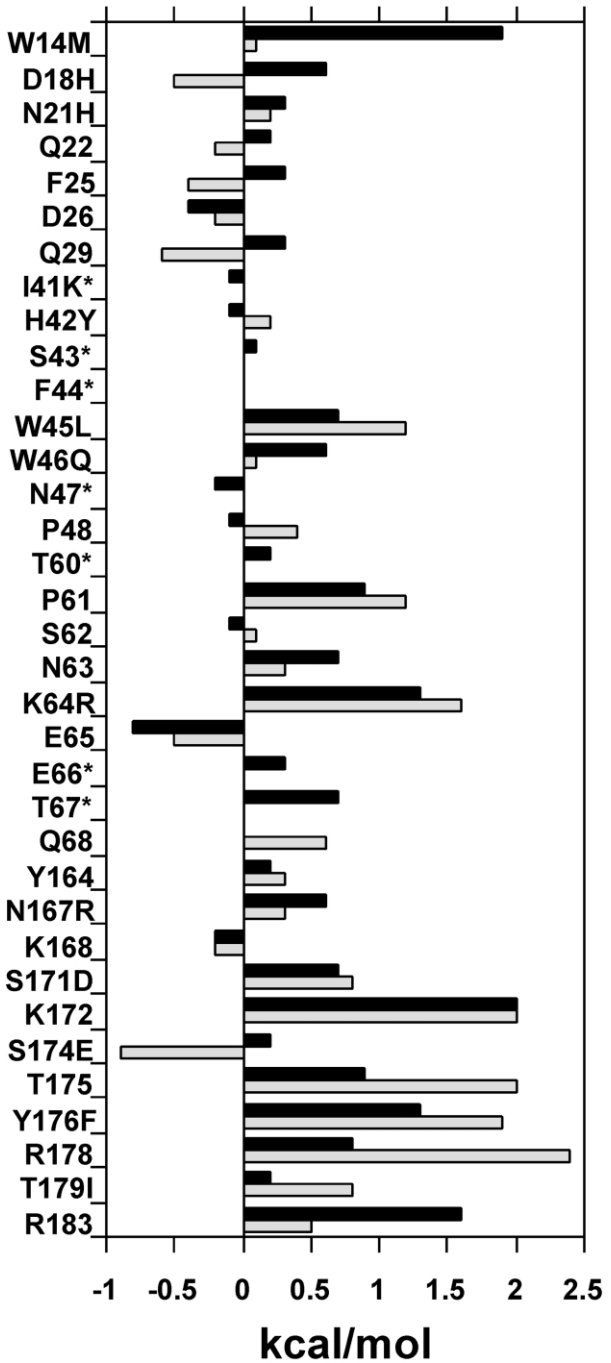

Figure 2. $\Delta\Delta G_{Ala-wt}$ values measuring the effects of alanine substitutions on site 1 binding affinity for the hGHR. Values for hGH_v (black bars) are from Table 1, while values for hGH_{wt} (gray bars) are from Cunningham and Wells.[6] The hGH_v residue is denoted by the single-letter code for amino acid, followed by a number denoting its position in the hGH sequence, and in positions where the sequences of hGH_v and hGH_{wt} differ, the corresponding residue in hGH_{wt} (e.g. W14M denotes position 14 which is Trp or Met in hGH_v or hGH_{wt}, respectively). Asterisks (*) denote residues which were not tested in the hGH_{wt} analysis.

(Figure 1A) and the wt molecule (Figure 1B) have a somewhat different distribution of their functionally most important residues. While most of the residues that contributed binding energy to hGH_{wt} site 1 remain important, the hGH_v site 1 binding epitope is expanded due to increased contributions from Arg183 on one side and Trp14, Asp18 and Arg167 on the other side. Furthermore, the hGH_v site 1 binding interface contains significantly fewer neutral or negative contributors to binding energy than does the wt interface (colored green and blue in Figure 1A and B).

Altered character of bystander and inhibitory residues

A significant portion of the hGH_{wt} site 1 interface is comprised of bystander residues (residues that make contact with the receptor but have little energetic role in binding) and inhibitory residues that actually have a small, but measurable negative effect on binding. A distinct trend in the hGH_v epitope is the reduced number of negative interactions. Of the five inhibitory residues in hGH_{wt} (colored blue in Figure 1B), only one (Glu65) has a negative effect on the binding interaction between hGH_v and the hGHR. Two of these residues (H18D and E174S) were mutated during the affinity maturation process, and these residues are notable because they play an essential role in the obligatory receptor-specific zinc-binding site formed at the interface of the hGH_{wt}–hPRLR interaction. The H18D, H21N and E174S mutations in hGH_v produce a set of new H-bonding interactions for Asp18 and Asn21 with the hGHR,[23] but they also result in the elimination of the zinc-binding site. Thus, the mutations and structural changes in this region create a variant site 1 that is highly optimized for binding to the hGHR but is no longer capable of binding to the hPRLR.[22] In contrast, the hGH_{wt} site 1 represents a structural compromise that can accommodate both the hGHR and the hPRLR with affinities that are sub-optimal yet sufficient for biological activity.

To better visualize the changes in importance of each analyzed hGH_v residue relative to hGH_{wt}, a residue by residue binding energy difference surface was calculated by subtracting $\Delta\Delta G_{Ala-wt}$ for hGH_{wt} from $\Delta\Delta G_{Ala-wt}$ for hGH_v (Figure 1(C)). The mapped surface shows a rather complex rearrangement of the functionally important residues in hGH_v, and emphasizes a cluster of three closely packed mutant hGH_v residues (Trp14, Asp18 and Ser174) that are significantly more important than their corresponding hGH_{wt} antecedents (Met14, His18 and Glu174). Interestingly, a fourth residue that gains significantly in importance (Arg183) was not mutated in the affinity maturation process, suggesting that its greatly increased influence has been generated through synergy with mutations at other positions.

It is notable that these two functionally evolved areas flank the original hGH_{wt} hot spot. Arg183 is

proximal to Thr179, while Trp14, Asp18 and Ser174 form a patch that borders Arg178 and Thr175 (Figure 1C). In addition, the side-chain of Arg183 acts as a scaffold that makes three H-bonds and structurally orders loop-60, which contains the important receptor-binding residue Lys64. While the original hGH_{wt} hot spot remains important for hGH_v site 1 binding, its contribution is significantly muted (colored blue in Figure 1C), but this attenuation is apparently counter-balanced by the increased functional importance of the flanking residues in hGH_v.

The site 1 binding energy surface of hGH_v indicates an additional small satellite of binding energy residing in the independently affinity-matured mini-helix; the main contributors are two tryptophan residues, Trp45 and Trp46. The main chain in the mini-helix region undergoes displacements of up to 8 Å between the unbound and bound forms of hGH_v,[27] indicating that the phage selection involved both sequence and conformational factors. The tryptophan residues in hGH_v move by about 7 Å upon binding, and they form a sandwich-like ring packing interaction with Trp76 of the hGHR. Interestingly, the residue at position 45 was also functionally important in the original hGH_{wt} site 1 interaction, even though the Leu45 in hGH_{wt} makes little direct contact with the receptor. Its role appears to be in forming a packing environment that positions the side-chain of Lys41 to form a salt-bridge with Glu127 of the receptor. It is noteworthy that, when the sum of the binding energy values for the hGH_v mini-helix is compared to that of the hGH_{wt} mini-helix, the optimized region does not appear to be more important (at least in the context of the other affinity-matured hormone residues). This suggests a negative cooperativity for combining this region with the other independently optimized mutations. This has been confirmed by alanine-scanning data showing that the energetic contributions of the individual residues in the context of the mini-helix mutations alone are greater than when they are combined with the mutations selected from the other libraries (B. Bernat and A.A.K., unpublished results).

Non-additivity effects from combining independently selected mutations

While many site-directed mutagenesis experiments have demonstrated that additive interactions dominate most protein–protein interactions,[6,7,30] cooperative intramolecular interactions can give rise to non-additivity.[31,32] In particular, the strategy used to affinity mature hGH_v site 1 may have favored the evolution of intramolecular cooperativity. The simultaneous randomization of proximal sites in each library could produce binding solutions that rely on positive cooperativity between spatially clustered mutations. Conversely, negative cooperativity could arise as a result of combining mutations from independent libraries. Furthermore, the affinity maturation of any of the individual libraries may have influenced indirectly other groups that were not part of the mutagenesis scheme. This may in part be the cause of the altered roles of several bystander and inhibitory residues in hGH_v which gained importance without being mutated themselves. The most conspicuous example is Arg183, a scaffold residue that orients a principal section of a binding loop. However, we note that, while it is possible to rationalize the importance of the Arg183 contributions to receptor binding in hGH_v, it is not clear why this group is apparently energetically less important in the hGH_{wt} interaction, given that it plays a similar scaffolding role in both molecules. In addition, the reduced functional contributions of several key residues in the original hGH_{wt} hot spot (Thr175, Tyr176, Arg178 and Thr179) may be caused by sub-optimal binding interactions that are necessitated by other regions of hGH_v that gain functional importance (Arg183, Trp14, Asp18 and Ser174).

Conclusions and Implications

The improved affinity of hGH_v site 1 resulted from the combination of four independent affinity maturation experiments. In each experiment, mutants contained in a localized region were selected for improved affinity for the hGHR. The mutations obtained by each of these selections were then consolidated to produce hGH_v. In such a procedure, there is no reason to believe that there would be any binding synergy between the individual mutated regions; on the contrary, one might expect that negative cooperativity might be introduced by the juxtaposition of independently evolved interactions. Our analysis provides several significant conclusions and also suggests further questions for investigation.

(1) The ~400-fold improvement in hGH_v site 1 binding affinity was not achieved through minor adjustments of existing interactions in the hGH_{wt} site 1. Previous structural studies revealed wholesale reconfiguration of many of the original binding elements;[23] the character of many mutant residues was quite different from the wt groups, and this forced wt salt-bridges and H-bonds to be broken and new ones to be formed. As a result, the binding energy distribution in the hGH_v site 1 interface is significantly different from that in hGH_{wt}. The contributions of residues in the original binding hot spot have been attenuated and additional interactions have been added on the periphery, thus resulting in an expanded and diffused hot spot.

(2) Most of the residues that inhibited binding in the wt interface have been replaced by residues that make either a positive contribution or have a null effect in the affinity-matured interface. As a result, hGH_v has improved affinity for the hGHR, but has lost the ability to bind to the hPRLR,

because some of the wt residues that inhibited binding to the hGHR were crucial for binding to the hPRLR.

(3) The *in vitro* evolution of hGH_v proceeded under fundamentally different principles from those believed to have governed the evolution of hGH_{wt} (at least over the last several million years). While there was a time-period, around the split between old and new-world monkeys, when primate growth hormones underwent major mutation,[33] even the fastest evolving proteins evolve through the accumulation of amino acid changes allowed by single base-pair changes (excluding extensive mutations generated by DNA shuffling). The *in vitro* evolution of hGH_v was not encumbered by this limitation; on the contrary, all possible combinations of the natural amino acids were simultaneously sampled at multiple, proximal sites. The simultaneous introduction of several spatially clustered changes can give rise to cooperative effects within the artificially evolved interface, and this contrasts with the observed properties of many single-site mutations which are characterized by the predominance of additive effects.[30] While the existence of cooperative effects remains to be demonstrated by experiments that are ongoing (B. Bernat and A.A.K., unpublished results), we speculate that the high degree of structural plasticity and the large-scale changes in binding energetics in hGH_v site 1 may, in part, be due to the introduction of cooperativity during the *in vitro* evolution process.

(4) The structural analysis of hGH_v complexed to two copies of the hGHR ECD did not pinpoint specific interactions responsible for the increased affinity of the hGHR ECD for site 1 of hGH_v.[23] Our mutational analysis indicates that, to a large extent, this may be due to the fact that improved affinity results from numerous small effects. In addition, it is possible that some of the improvements in binding affinity are not generated by effects within the bound state, but rather, some of the mutations may have unfavorable effects on the unbound hormone molecule. Thus, structural rationales that explain the improved affinity of hGH_v will not be found by studying only the interactions within the bound complex. It is important to appreciate the possible contributions from the increased ease of desolvation of the unbound hormone.

Materials and Methods

Materials

Enzymes and M13-KO7 helper phage were from New England Biolabs. Maxisorp immunoplates were from NUNC (Roskilde, Denmark). *Escherichia coli* XL1-Blue was from Stratagene. Bovine serum albumin (BSA) and Tween-20 were from Sigma.

Oligonucleotides

Equimolar DNA degeneracies are represented in the IUB code (K, G/T; M, A/C; R, A/G; S, G/C; Y, C/T). Degenerate codons are shown in bold. The following mutagenic oligonucleotides were used for library constructions to randomize residues in helix-1 (*H1*); mini-helix (*MH*); loop-60 (*L60*) and helix-4 (*H4*):

H1: CCA CTA AGT CGA CTA GCT GAT AAC GCT **KSG** CTT CGG GCC **GMT** CGT CTT **RMC SMA** CTA GCC **KYT GMT** ACG TAC **SMA** GAG TTT GAA GAG GCC TAT

MH: ATC CCC AAG GAA CAG **RYT SMT KCC KYT KSG KSG RMC SCA** CAG ACC TCC CTC TGT

L60: TTC TCA GAA TCG ATT CCG **RCT SCA KCC RMC RMA GMA GMA RCT SMA** CAG AAA TCC AAC CTA GAG

H4: AAG AAC TAC GGG CTG CTC **KMT** TGC TTC **RMC RMA** GAC ATG **KCC RMA** GTC **KCC RCT KMT** CTG **SST RCT** GTG CAG TGC **SST** TCT GTG GAG GGC AGC

Shotgun scanning library construction

A library of hGH_v site 1 variants was constructed using previously described methods[28] and a modified version of a phagemid designed for the display of hGH on the surface of M13 bacteriophage.[24] The library was designed to simultaneously replace 35 codons with degenerate "shotgun alanine-scanning" codons; for each position, a degenerate codon was designed to encode the hGH_v sequence and Ala (and two other amino acid residues at some positions) with equal probability, as described previously.[24] A "stop template" version of the hGH display phagemid (containing TAA stop codons in positions to be mutated) was used as the template for the Kunkel mutagenesis method[34] with four mutagenic oligonucleotides (*H1*, *MH*, *L60*, and *H4*) designed to simultaneously repair the stop codons and introduce mutations at the desired sites. The mutagenesis reaction was electroporated into *E. coli* SS320[28] to produce a library containing 2.0×10^{10} unique members. After overnight growth at 37 °C, in 2YT medium supplemented with 50 μg/ml carbenicillin and M13-KO7 helper phage, library phage were concentrated by precipitation with PEG/NaCl and resuspended in PBS, 0.5% (w/v) BSA, 0.1% (v/v) Tween-20, as described previously.[28]

Library sorting and binding assays

Maxisorp immunoplates (96-well) were coated overnight at 4 °C with capture target (either the hGHR ECD or anti-hGH monoclonal antibody 3F6-B1.4B1[29] at 5 μg/ml) and blocked for two hours with BSA. Phage solutions from the libraries described above (10^{12} phage/ml) were added to the coated immunoplates, incubated for two hours at 25 °C to allow for binding, and washed 12 times with PBS, 0.05% Tween-20. Bound phage particles were eluted with 0.1 M HCl for ten minutes and the eluant was neutralized with 1.0 M Tris base. Eluted phage were amplified in *E. coli* XL1-blue with M13-KO7 helper phage and used for further rounds of selection.

Individual clones from each round of selection were grown in a 96-well format, and the culture supernatants were used directly in phage ELISAs[28] to detect phage that bound to either hGHR ECD or anti-hGH antibody. After two rounds of selection, 50% of the clones from the hGHR ECD selection and 100% of the clones from

the anti-hGH antibody selection exhibited positive phage ELISA signals at least twofold greater than signals on control plates coated with BSA. These positive clones were subjected to DNA sequence analysis.

DNA sequencing and analysis

Culture supernatants containing phage particles were used as templates for PCRs that amplified the hGH gene. The PCR primers were designed to add M13(−21) and M13R universal sequencing primers at either end of the amplified fragment, thus facilitating the use of these primers in sequencing reactions. Amplified DNA fragments were sequenced using Big-Dye terminator sequencing reactions with the M13(−21) universal sequencing primer, and a single sequencing reaction was sufficient to read all mutated positions. The reactions were performed in a 96-well format and analyzed on an ABI Prism 3700 96-capillary DNA analyzer (PE Biosystems, Foster City, CA).

The sequences were analyzed with the program SGCOUNT as described previously.[24] SGCOUNT aligned each DNA sequence against the hGH_v DNA sequence by using a Needleman–Wunch pair-wise alignment algorithm, translated each aligned sequence of acceptable quality, and tabulated the occurrence of each natural amino acid at each position. In addition, SGCOUNT reported the occurrence of any sequences containing identical amino acid residues at all mutated positions (siblings). For the antibody selection, 91 clones were sequenced and no siblings were observed. For the hGHR selection, 183 clones were sequenced and 167 of these were unique, while eight sequences occurred twice each.

Analysis of shotgun alanine-scanning data

The DNA sequence data from the hGHR and antibody selections were used to determine $\Delta\Delta G_{Ala-wt}$ values for each side-chain mutation, as described previously.[24] For each mutated position and each selection, the wt/Ala ratio was determined and substituted for the $K_{a,wt}/K_{a,Ala}$ ratio in the standard equation:

$$\Delta\Delta G = RT \ln(K_{a,wt}/K_{a,Ala}) = RT \ln(\text{wt/Ala})$$

this calculation provided a measure of the effect of each alanine substitution on each selection as a change in free energy relative to that of the wt. To obtain an estimate of the contribution to binding free energy attributable to each side-chain ($\Delta\Delta G_{Ala-wt}$), we used the $\Delta\Delta G$ values from the antibody selection to correct the $\Delta\Delta G$ values from the hGHR selection for effects on display level or biases in the naïve library, as follows:

$$\Delta\Delta G_{Ala-wt} = \Delta\Delta G_{hGHR} - \Delta\Delta G_{\alpha}$$

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