Engineering an interfacial zinc site to increase hormone–receptor affinity

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Background: Human growth hormone (hGH) binds to both the hGH and human prolactin (hPRL) receptors. Binding to the hPRL receptor, however, is ~50-fold tighter and requires a single Zn^{2+} cation, unlike binding of hGH to the hGH receptor. Previous mutational studies have identified putative ligands from hGH and the hPRL receptor responsible for coordinating the interfacial Zn^{2+}.

Results: One of these ligands was introduced at a structurally analogous site in the extracellular domain of the hGH receptor by mutating Asn218 to His, and the resulting mutant protein showed a 20-fold increase in hGH binding in the presence of $ZnCl_2$. Alanine-scanning mutagenesis showed that the binding site on hGH for the Asn218→His hGH receptor in the presence of Zn^{2+} resembled that for the hPRL receptor.

Conclusions: It is possible to introduce the metal-binding site from the hPRL receptor into the homologous hGH receptor. More generally, these studies indicate that affinity between two proteins may be enhanced by design of an interfacial metal-binding site.

Chemistry & Biology September 1994, **1**:25–30

Key words: growth hormone, prolactin, protein engineering, protein–protein interactions, zinc sites

Introduction

The design of metalloproteins and the incorporation of metal-binding sites into protein frameworks has received considerable attention in recent years (see [1,2] for recent reviews). Engineered metal-binding sites have been useful in attempts to stabilize proteins by making loops more rigid (for example, see [3,4]) or by bridging α-helices [5,6]. A metal-ion binding site has also been introduced into an antibody domain by rational protein design [7], and metal sites have been used to assist in protein purification (reviewed in [8]).

We wondered whether it was possible to use a metal-binding site to enhance the affinity of a protein–protein interaction. The zinc-mediated interaction of human growth hormone (hGH) with the extracellular domain of the human prolactin receptor (hPRLbp) is a naturally occurring example of such an interaction [9]. In the absence of Zn^{2+}, hGH binds to hPRLbp with relatively low affinity (K_d = 270 nM). In the presence of 50 μM $ZnCl_2$, however, the binding affinity increases about 8 000-fold (K_d = 33 pM). In contrast, zinc is not required for the binding of hGH to the extracellular domain of the hGH receptor (hGHbp).

Scanning mutational studies [9,10] indicated that the ligands for binding zinc are shared between hGH and hPRLbp. In particular, His188 on hPRLbp was shown to be important in binding Zn^{2+}. Based on sequence alignments, the corresponding residue in hGHbp is Asn218. In view of the dramatic increase in binding affinity of the hGH–hPRLbp interaction in the presence of zinc, we wondered whether a similar zinc-binding site could be introduced into the hGH–hGHbp interface. Here we report the design and characterization of the hGHbp variant, Asn218→His (N218H), and show that it has increased affinity for hGH in the presence of Zn^{2+}. We also present the functional mapping of the residues on hGH responsible for binding to the mutant hGHbp and discuss the possible mechanism of Zn^{2+}-mediated binding enhancement in the light of the recently determined structure of hGH in complex with hPRLbp in the presence of Zn^{2+} (W. Somers, A. deVos, A. Kossiakoff, personal communication).

Results and discussion

Design, construction and expression of mutants

Analysis of the sequences of growth hormone and prolactin receptors from different species shows that His188 in the prolactin receptor is conserved, whereas the homologous residue in the growth hormone receptor is Asn218 (human) or Ser (rabbit, mouse and rat). Furthermore, in the three-dimensional structure of the hGH–$(hGHbp)_2$ co-complex [11] the side chain of receptor residue Asn218 is buried in the hormone–receptor interface between His18, His21 and Glu174 of the hormone (Fig. 1). Asn218 is one of the most deeply buried residues in the interface between hGH and hGHbp, losing more than 100 $Å^2$ of solvent-accessible surface upon binding of the hormone. We therefore reasoned that it may be possible to recruit zinc dependency into the hGH–hGHbp interaction by changing Asn218 to His.

Modelling of the hGH–hGHbp structure indicates that His218 in the receptor could possibly coordinate a Zn^{2+} ion along with several side chains from hGH. Indeed, one of the heavy metal derivatives used to solve the crystal structure

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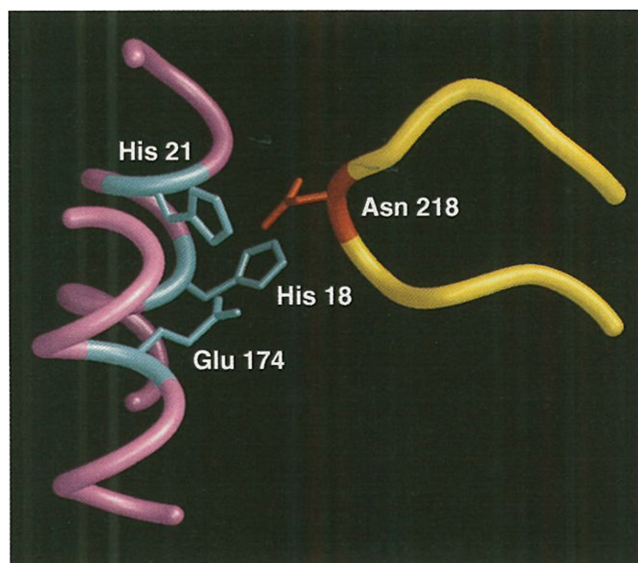

Fig. 1. Structure of the region around Asn218 in hGHbp in complex with hGH [11]. Helices from hGH are in pink and the loop containing Asn218 in hGHbp is in yellow. Asn218 from hGHbp is shown in red, and the putative Zn^{2+} ligands from hGHbp are in blue.

(K_2AuCl_4) has a gold ion in this region (A. deVos, personal communication). As a control, and to investigate the functional role of Asn218 in the wild-type hGH–hGHbp interaction, we constructed a mutant hGHbp in which Asn218 was replaced by Ala. The hGHbp mutants N218A and N218H were constructed using site-directed mutagenesis and were expressed and purified as described in Materials and methods. The purified proteins were analyzed by SDS polyacrylamide gel electrophoresis and found to be >90 % pure (data not shown).

Binding of hGH to hGH receptor mutants

In the absence of Zn^{2+}, the N218A substitution in hGHbp has little effect on hGH affinity (Table 1). This is remarkable considering that Asn218 is the second most deeply buried residue in the X-ray crystal structure, but not unprecedented in light of recent mutational analysis of hGH, which shows that over half of the side chains that contact the receptor in the crystal structure can be singly mutated to Ala with little or no effect on affinity [12]. Kinetic analysis by surface plasmon resonance using the BIAcore system showed that there is less than a two-fold increase in off-rate and a negligible effect on the on-rate for the binding of N218A hGHbp to hGH in the absence of Zn^{2+} (data not shown). Even in the case of the N218H substitution, there is no disruption of binding in the absence of Zn^{2+}.

Table 1. K_d values for the interaction of hGH with wild-type, N218H and N218A hGHbp in the presence and absence of Zn^{2+}.

	K_d –Zn (nM)	K_d +Zn (nM)
WT hGHbp	1.05 ± 0.2	3.0 ± 1.0
N218A hGHbp	1.25 ± 0.41	0.31 ± 0.043
N218H hGHbp	0.93 ± 0.13	0.046 ± 0.014

Values were determined by radioimmunoassay with antibody MAb5, which inhibits receptor dimerization and only allows formation of a 1:1 hormone–receptor complex. Mutations are designated by the wild-type residue that is mutated (single letter amino acid code) followed by its sequence position and mutant residue. For example, N218A represents a mutation where asparagine 218 is converted to alanine. WT, wild-type.

In the presence of 50 μM $ZnCl_2$, the binding of hGH to wild-type hGHbp is slightly weaker than in the absence of Zn^{2+}. The affinity of the N218A mutant is slightly higher in the presence of Zn^{2+}, however, suggesting the possibility that weak binding of Zn^{2+} in the wild-type hGH–hGHbp complex is sterically disruptive, and that this disruption can be alleviated by the N218H mutation. The affinity of the N218H mutant increases approximately 20-fold in the presence of 50 μM $ZnCl_2$, with the result that it binds to hGH almost as tightly as does the hPRL receptor in the presence of similar concentrations of Zn^{2+}. Moreover, the zinc concentration required for half-maximal binding of hGH to either hPRLbp or N218H hGHbp was similar (10–20 μM $ZnCl_2$), and in both systems binding was nearly maximal at 50 μM $ZnCl_2$ ([9], data not shown).

We also studied binding of hGH to wild-type hGHbp and N218H hGHbp in the presence and absence of Zn^{2+} using analysis with the BIAcore system (Table 2).

Table 2. BIAcore data for binding of hGH to immobilized wild-type hGHbp and N218H hGHbp in the presence and absence of Zn^{2+}.

	k_{on} $M^{-1}s^{-1}$		k_{off} s^{-1}	
	$-ZnCl_2$	$+ZnCl_2$	$-ZnCl_2$	$+ZnCl_2$
WT hGHbp	3.0×10^5	ND	2.7×10^{-4}	ND
N218H hGHbp	2.7×10^5	2.3×10^5	3.2×10^{-4}	1.2×10^{-5}*

The data for wild-type hGHbp binding to hGH are taken from [12]; ND, not determined. *Calculated from ($K_d \cdot k_{on}$).

In the absence of Zn^{2+} the on and off rates for wild-type and N218H hGHbp were very similar. In the presence of Zn^{2+}, the on-rate for N218H hGHbp was hardly changed; however, the off-rate was decreased about 10-fold, and could not be reliably measured by the instrument. We therefore report a value calculated from [$K_d \cdot k_{on}$]. The decrease in off-rate is consistent with previous protein-engineering studies in which affinity enhancements were found largely to affect off rates and not on rates [12–14].

Mapping the binding site on hGH using alanine-scanning mutagenesis

The hGHbp and hPRLbp receptors have overlapping but distinctly different binding sites on hGH [10]. We therefore wondered whether the N218H hGHbp mutant

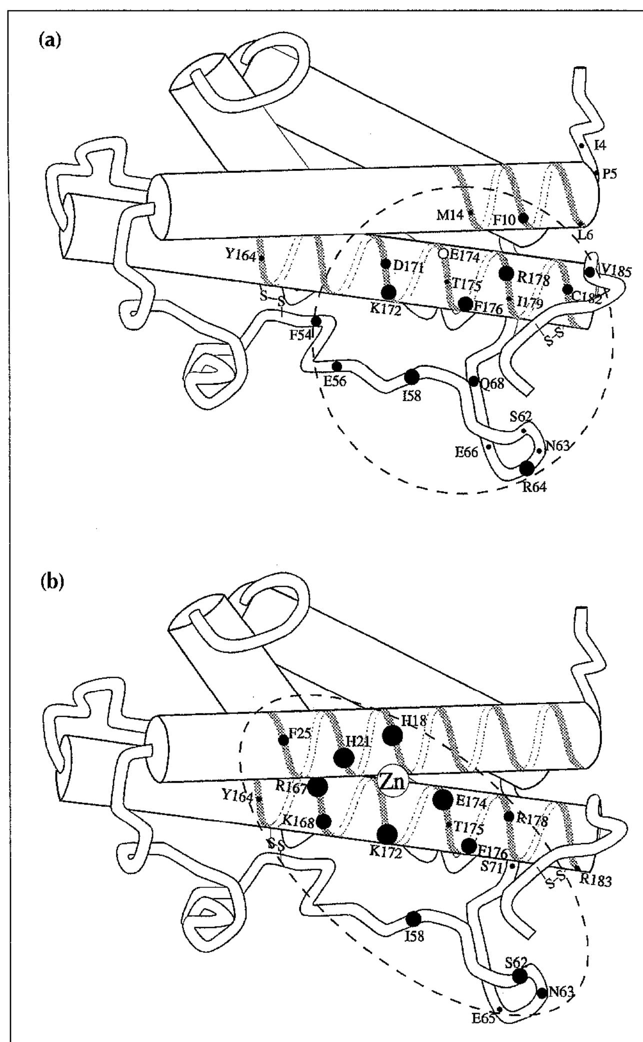

Fig. 2. Receptor-binding determinants identified **(a)** for hGHbp and **(b)** for hPRLbp binding sites by alanine-scanning mutagenesis of hGH [9,10,15].

Table 3. K_d(mut)/K_d(WT) for hGH mutants binding to wild-type hGHbp and N218H hGHbp.

Mutant	±Zn	WT hGHbp K_d(mut)/K_d(WT)	N218H hGHbp K_d(mut)/K_d(WT)	ΔΔΔG kcal mol^{-1}
E56A	+	1.6±0.7	1.2±0.31	–0.12
	–	4.1*	2.5±0.6	–0.29
R64A	+	18.0±14.6	6.2±2.52	–0.58
	–	21*	26.5±6.9	–0.14
D171A	+	4.9±1.9	1.8±0.7	–0.54
	–	7.1*	6.8±1.7	–0.02
H18A	+	0.18±0.09	0.4±0.1	0.65
	–	1.6*	0.35±0.09	–0.86
H21A	+	0.15±0.11	0.4±0.1	0.76
	–	0.33*	0.19±0.07	–0.29
E174A	+	0.036±0.020	1.8±0.7	2.3
	–	0.22*	0.23±0.07	–0.04
R167A	+	0.93±0.48	2.4±0.6	0.63
	–	0.75*	1.03±0.25	0.18
K168A	+	0.18±0.09	3.4±1.4	1.84
	–	1.1*	1.14±0.25	0
R64K/H21A/	+	0.03±0.01	1.0±0.3	2.13
E174A	–	0.03†	0.03±0.01	0.65

Values were determined by radioimmunoassay using antibody MAb5. ΔΔG for binding to wild-type or N218H variant hGHbp, defined as $\Delta\Delta G = RT\ln(K_d(\text{hGH mutant})/K_d(\text{WT hGH}))$, was used to calculate $\Delta\Delta\Delta G = \Delta\Delta G_{(N218H\ hGHbp)} - \Delta\Delta G_{(wt\ hGHbp)}$. Hence, positive values of ΔΔΔG indicate that the mutant hGH has a more disruptive (or less enhancing) effect on N218H hGHbp binding than on wild-type hGHbp binding. *K_d(mut)/K_d(WT) for single hGH alanine mutants calculated from [10]. †K_d(mut)/K_d(WT) for R64K/H21A/E174A mutant calculated from [15]. Errors in single K_d values for these measurements were estimated as ±25 %.

retained the hGHbp mode of binding to hGH or had a novel hGH binding site. The hGH residues that were found to affect either hGHbp binding or hPRLbp binding in alanine-scanning studies are shown in Fig. 2. In addition, the hGH mutant R64K/H21A/E174A has been shown to have a greatly enhanced specificity for binding to hGHbp compared with hPRLbp [15]. To examine further the nature of the interface between N218H hGHbp and hGH, we used hGH mutants in which residues at the receptor interface were replaced with Ala [16]. We determined the values of K_d(mut)/K_d(WT) for binding of these analogs to N218H hGHbp in the presence and absence of 50 μM $ZnCl_2$ (Table 3).

In the absence of Zn^{2+}, there is little difference between the binding of most of the Ala mutants of hGH to wild-type hGHbp or to the N218H variant (Fig. 3a). For example, hGH mutants that are known to have a reduced affinity for the wild-type receptor (E56A, R64A and D171A) also have a reduced affinity for binding to N218H hGHbp. Moreover, the hGH mutants H18A, H21A, E174A, R167A and K168A are known to exhibit decreased binding to hPRLbp, but their binding to hGHbp is not much decreased (and for some mutants is even enhanced). Again, the same effects are observed for binding to N218H hGHbp. The only anomaly is H18A hGH, which binds slightly more weakly to wild-type hGHbp, but somewhat more tightly to N218H hGHbp, than does wild-type hGH. This observation is not surprising, as the crystal structure of the hGH–hGHbp complex shows that hGH residue His18 is in direct contact with Asn218 in the receptor. Also, the triple mutant R64K/H21A/E174A, which is extremely selective for binding to wild-type hGHbp compared with hPRLbp, has the same affinity for wild-type hGHbp as for N218H hGHbp.

In the presence of Zn^{2+}, some of the alanine mutants of hGH bind with very different affinities to wild-type and N218H hGHbp (Fig. 3b). Generally, hGH mutants that are reduced in affinity for hGHbp but not hPRLbp (E56A, R64A and D171A; Fig. 2) are not as reduced in affinity when binding to N218H hGHbp. Similarly, the hGH mutants R167A and K168A (which have reduced

affinity for hPRLbp but are distant from the site of the Zn^{2+} ligand) show decreased binding to N218H hGHbp but not to wild-type hGHbp. In fact, the K168A mutation somewhat enhances binding to wild-type hGHbp in the presence of Zn^{2+}. These results are qualitatively consistent with hGH binding to the N218H variant as it does to the hPRL receptor; however, there are some important quantitative differences. For example, mutating any one of the putative Zn^{2+} ligands (His18, His21 or Glu174) to alanine has little effect on binding of hGH to N218H hGHbp in the presence of Zn^{2+}. Only E174A shows a small (2-fold) decrease in binding. The same hGH mutants all have a higher affinity for wild-type hGHbp in the presence of Zn^{2+}. It is possible that the mode of binding shifts for these mutants, resulting in other compensating interactions. For example, Asp171 in hGH is close to the proposed Zn^{2+} coordination site [11] and may coordinate Zn^{2+} when one of the other ligands on hGH is mutated to Ala. Whatever the explanation, the fact that the affinity of the N218H hGHbp variant is much higher for the H18A, H21A and E174A hGH mutants in the presence of Zn^{2+} indicates that Zn^{2+} coordination is still able to enhance the affinity of the protein–protein interaction. Moreover, the hGHbp-specific triple mutant R64K/H21A/E174A hGH binds more tightly than wild-type hGH to the N218H receptor in the absence of Zn^{2+} but has the same affinity as wild-type hGH in the presence of Zn^{2+}. This variant binds much more tightly than wild-type hGH to wild-type hGHbp in the presence and absence of Zn^{2+}. Thus, in this case it seems that simultaneously introducing the mutations R64K, H21A and E174A into hGH diminishes the Zn^{2+}-mediated binding to hGHbp but enhances the Zn^{2+}-independent binding (Table 3).

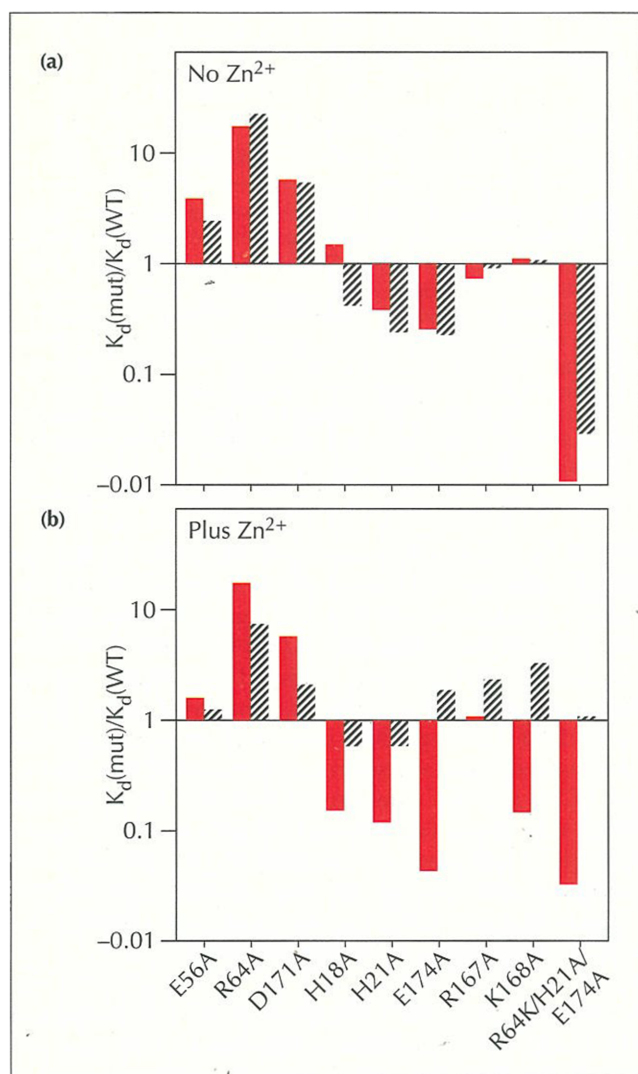

Fig. 3. Effect of hGH mutations on binding to wild-type hGHbp (solid red bars) and N218H hGHbp (hatched bars) in **(a)** the absence and **(b)** the presence of Zn^{2+}, represented as K_d(mut)/K_d(WT).

Another way to express the change in binding specificity for the N218H mutant relative to the wild-type hGHbp is to calculate the free energy change between them on binding the hGH mutants (Fig. 4). In this case, a positive value of $\Delta\Delta\Delta G$ (as defined in Table 3) indicates that a particular hGH mutant has a more disruptive effect (relative to hGH) on binding to the N218H variant compared with binding to wild-type hGHbp. From this analysis, it is clear that in the absence of Zn^{2+} (Fig. 4a) both wild-type and N218H hGHbp bind the hGH mutants with similar affinities, in other words, $\Delta\Delta\Delta G$ is small (with the exception of the H18A mutant, as discussed above). In the presence of Zn^{2+} (Fig. 4b), the hGH mutants that show reduced affinity for wild-type hGHbp (E56A, R64A and D171A) are not as much reduced in affinity for the N218H hGHbp variant (negative $\Delta\Delta\Delta G$). Conversely, $\Delta\Delta\Delta G$ is positive for those hGH mutants that show reduced affinity for hPRLbp (H18A, H21A, E174A, R167A and K168A), indicating that hGH binding to wild-type hGHbp is significantly less disrupted (more enhanced) by these mutations than is binding to N218H hGHbp. Hence, the mutagenesis data provide evidence that, in the absence of Zn^{2+}, the binding site for N218H hGHbp on hGH is similar to that for the wild-type hGHbp. On addition of Zn^{2+}, the binding site for N218H hGHbp on hGH is more like that of hPRLbp than that of hGHbp.

Implications for the design of interfacial metal-binding sites

By means of a single amino acid substitution (N218H) in hGHbp, we have recruited a zinc ligand into the hGH–hGHbp interface. Although Asn218 is deeply buried in the hormone–receptor interface, it makes a negligible contribution to overall binding energy as measured by RIA and BIAcore analysis of the N218A mutant. The K_d for the N218H mutant receptor binding to hGH is about 65 times lower than that for wild-type hGHbp binding to hGH in the presence of Zn^{2+}, and about 20 times lower than that of hGH binding to N218H hGHbp in the absence of Zn^{2+}. Although the Zn^{2+}-mediated enhancement is not as great as that observed for hPRL binding to the hPRL receptor, the absolute K_d value is similar.

Recently, the structure of hGH in complex with hPRLbp in the presence of Zn^{2+} has been solved (W. Somers, A. deVos, A. Kossiakoff, personal communication). This structure indicates that only two of the putative Zn^{2+} ligands from hGH, His18 and Glu174, are in direct contact with the Zn^{2+} ion, and that Asp187 as well as His188 from the hPRLbp are involved in coordinating the metal ion. We have constructed a mutant hGHbp that incorporates both of the Zn^{2+} ligands from the hPRLbp (R217D/N218H) and find that it binds hGH in the presence of Zn^{2+} with a K_d of ~25 pM (data not shown), only two-fold lower than that for the N218H mutant. In view of the recent structural information, it is perhaps remarkable that the single N218H mutant exhibits such a pronounced effect in the absence of the R217D substitution. We hypothesize that several alternative Zn^{2+} coordination schemes may be possible at this region of the hormone–receptor interface, involving His18, His21, Glu174 and/or possibly Asp171 from the hormone. Further mutational analysis and structural studies will be needed to sort out these alternative coordination schemes.

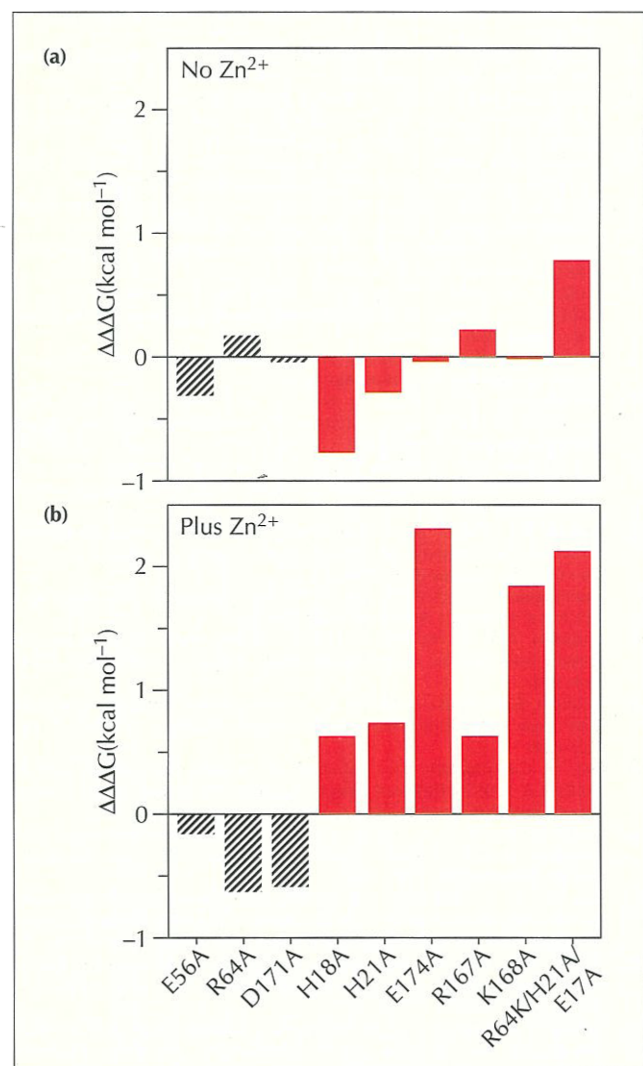

Fig. 4. Difference between binding of mutant hGH proteins to wild-type and N218H hGH receptors in **(a)** the absence and **(b)** the presence of Zn^{2+}. Hatched bars indicate mutants that show reduced affinity for wild-type hGHbp; solid red bars, mutants that show reduced affinity for hPRLbp. The graph shows $\Delta\Delta\Delta G = \Delta\Delta G_{N218H} - \Delta\Delta G_{WT}$, where $\Delta\Delta G = RT\ln(K_d(\text{hGH mutant})/K_d(\text{WT hGH}))$ for either the WT hGHbp or N218H hGHbp. Positive values of $\Delta\Delta\Delta G$ indicate that the hGH mutant has a more disruptive (or less enhancing) effect on N218H hGHbp binding compared to WT hGHbp binding. Conversely, negative values of $\Delta\Delta\Delta G$ indicate that binding of the mutant hGH to the N218H hGHbp is less disruptive (or more enhanced) compared to WT hGHbp.

Significance

Human growth hormone (hGH) binds to two cell surface receptors, the hGH and human prolactin (hPRL) receptors, with different results; while binding to the hGH receptor affects many biological processes, including linear bone growth and somatogenesis, binding to the human prolactin receptor affects mainly lactation and immune stimulation. The binding sites on hGH for these receptors are distinctly different, and hGH binding to the hPRL receptor is greatly enhanced in the presence of zinc, whereas hGH binding to the hGH receptor is zinc independent [10]. Zinc is important in many endocrine functions, and the zinc dependency of hGH binding to the hPRL receptor may explain some of the medical effects of zinc deficiency, including growth retardation and hyperprolactinemia.

Zinc-binding sites in proteins typically involve coordination by four ligands. In the hGH–hPRL receptor pair the zinc-coordinating ligands are shared between the hormone and the receptor. Earlier studies had shown that zinc binding at this site is essential for high-affinity hormone binding, and we were thus interested to see whether engineering a zinc coordination site in the extracellular domain of the hGH receptor (hGHbp) could also increase the affinity of the hGH–hPRLbp interaction in a zinc-dependent manner. Structural and sequence comparisons between the two proteins indicated that it might be possible to engineer a zinc-coordination ligand into hGHbp by changing a single amino acid. Thus, by engineering a single His residue into hGHbp, we created an interfacial zinc-binding site, which resulted in a nearly 100-fold improvement in the binding affinity.

Interestingly, recent structural studies on the complex of hGH with hPRLbp indicate that the original model of zinc coordination in this complex was not completely accurate, in that it now seems that not one but two of the zinc-coordinating ligands come from the hPRL receptor. Thus the explanation for the successful zinc coordination in our engineered complex remains to be determined in detail. Nevertheless, these studies suggest that improving protein–protein interactions by incorporating a zinc-binding site between protein partners is feasible, and that such a site can provide large

improvements in affinity in the presence of zinc. There are now numerous examples of proteins that have been successfully engineered with metal-binding sites [5–8], and thus it is reasonable to expect that interfacial zinc sites can be designed between proteins, providing that the placement of the ligands at the site does not sterically or electrostatically disrupt the interface.

Materials and methods

Materials

T4 polynucleotide kinase, T4 DNA ligase and T7 DNA polymerase were purchased from New England Biolabs or Gibco–BRL. Monoclonal anti-hGH receptor antibody MAb5 was obtained from Agen Biomedical Ltd. Materials for BIAcore biosensor analysis were obtained from Pharmacia Biosensor.

Preparation of hGHbp and hGH mutants

Mutants of hGHbp were made in the vector phGHr(1–238) [17]. Expression of hGHbp is directed by the *Escherichia coli* alkaline phosphatase (*phoA*) promoter and stII signal sequence [18]. The vector also contains an f1 origin, facilitating the production of single-stranded DNA for site-directed mutagenesis [19] and sequencing [20].

Expression and purification of mutant proteins was essentially as described for wild-type hGHbp [17,21]. The expression and purification of the hGH mutants used in alanine-scanning experiments has been described previously [16].

Binding assays for hGHbp

Binding affinities were measured using competitive displacement of [^{125}I]hGH and precipitation using an anti-hGHbp antibody (MAb5) as described [17,22], in a buffer containing either 50 μM $ZnCl_2$, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.1 % bovine serum albumin and 0.02 % sodium azide or 1 mM EDTA, 50 mM Tris (pH 7.5), 0.1 % bovine serum albumin and 0.02 % sodium azide.

Analysis of on and off rates was performed using surface plasmon resonance detection with the BIAcore system (Pharmacia). To couple mutant hGHbp to the biosensor chip, a cysteine residue was introduced in place of Ser201 in the receptor by site-directed mutagenesis. This residue is in the receptor–receptor interface [11]. Therefore, coupling hGHbp to the dextran matrix via this residue only allows formation of the 1:1 hGH:hGHbp complex [12]. The S201C mutant was expressed in *E. coli* and purified as described above, except that the purified protein was incubated with 1/39 volume of 1 M L-cysteine in 1 M $NaHCO_3$ at 4 °C for 2 h to deblock any intermolecular cysteine bonds and desalted by gel filtration on a PD10 column (Pharmacia). For coupling to the biosensor chip, the carboxy-dextran chip surface was activated with *n*-ethy-*n*-(3-diethylaminopropyl)-carbodiimide (EDC) and reacted with *n*-hydroxysuccinimide (NHS). The NHS-ester was then reacted with 2-(2-pyridinyldithio)-ethaneamine (PDEA). Unreacted NHS-ester groups were displaced by ethanolamine. The hGHbp variant was reacted with the matrix (at 10–50 μg ml^{-1} in 10 mM sodium acetate pH 5.0) until approximately 1 800 resonance units (RUs) were coupled. For determination of on rates, the binding profiles of hGH were measured at different concentrations ranging from 10 nM to 1 000 nM in an assay buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 0.05 % (w/v) Tween 20 containing either 1 mM EDTA or 50 μM $ZnCl_2$. The matrix was regenerated by washing for 20 s with 4.5 M $MgCl_2$. Off rate measurements were performed by saturating the chip with a high concentration (5–10 μM) of hGH. To minimize the effects of rebinding, only the initial portion of the dissociation curve was used for calculation. Both association and dissociation rates were determined using the manufacturer's software [23].

Acknowledgements: We thank Will Somers, Bart deVos and Tony Kossiakoff for making available crystallographic data prior to publication.

References

1. Berg, J.M. (1993). Metalloprotein design. *Curr. Opin. Struct. Biol.* **3**, 585–588.
2. Regan, L. (1993). The design of metal-binding sites in proteins. *Annu. Rev. Biophys. Biomol. Struct.* **22**, 257–287.
3. Pantoliano, M.W., *et al.*, & Bryan, P.L. (1988). The engineering of binding affinity at metal ion binding sites for the stabilization of proteins: subtilisin as a test case. *Biochemistry* **27**, 8311–8317.
4. Braxton, S. & Wells, J.A. (1992). Incorporation of a stabilizing Ca^{2+} binding loop into subtilisin BPN'. *Biochemistry* **31**, 7796–7801.
5. Regan, L. & Clarke, N.D. (1990). A tetrahedral zinc(II)-binding site introduced into a designed protein. *Biochemistry* **29**, 10878–10883.
6. Handel, T. & DeGrado, W.F. (1990). De novo design of a Zn^{2+} protein. *J. Am. Chem. Soc.* **112**, 6710–6712.
7. Pessi, A., Bianchi, E., Crameri, A., Venturini, S., Tramontano, A. & Sollazzo, M. (1993). A designed metal-binding protein with a novel fold. *Nature* **362**, 367–369.
8. Arnold, F.H. (1991). Metal-affinity separations: a new dimension in protein processing. *Biotechnology* **9**, 151–156
9. Cunningham, B.C., Bass, S., Fuh, G. & Wells, J.A. (1990). Zinc mediation of the binding of human growth hormone to the human prolactin receptor. *Science* **250**, 1709–1712.
10. Cunningham, B.C. & Wells, J.A. (1991). Rational design of receptor-specific variants of human growth hormone. *Proc. Natl. Acad. Sci. USA* **88**, 3407–3411.
11. DeVos, A., Ultsch, M. & Kossiakoff, A.A. (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306–312.
12. Cunningham, B.C. & Wells, J.A. (1993). Comparison of a structural and functional epitope. *J. Mol. Biol.* **234**, 554–563.
13. Lowman, H.B. & Wells, J.A. (1993). Affinity maturation of human growth hormone by monovalent phage display. *J. Mol. Biol.* **234**, 564–578.
14. Griffiths, A.D., *et al.*, & Winter, G. (1993). Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* **12**, 725–734.
15. Fuh, G., Cunningham, B.C., Fukunaga, R., Nagata, S., Goeddel, D.V. & Wells, J.A. (1992). Rational design of potent antagonists to the human growth hormone receptor. *Science* **256**, 1677–1680.
16. Cunningham, B.C. & Wells, J.A. (1989). High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* **244**, 1081–1085.
17. Fuh, G., *et al.*, & Wells, J.A. (1990). The human growth hormone receptor: secretion from *Escherichia coli* and disulfide bonding pattern of the extracellular binding domain. *J. Biol. Chem.* **265**, 3111–3115.
18. Chang, C.N., Rey, M., Bochner, B., Heyneker, H. & Gray, G. (1987). High-level secretion of human growth hormone by *Escherichia coli*. *Gene* **55**, 189–196.
19. Kunkel, T.A., Roberts, J.D. & Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.
20. Sanger, F., Nicklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
21. Bass, S.H., Mulkerrin, M.G. & Wells, J.A. (1991). A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. *Proc. Natl. Acad. Sci. USA* **88**, 4498–4502.
22. Spencer, S.A., Hammonds, R.G., Henzel, W.J., Rodriguez, H., Waters, M.J. & Wood, W.I. (1988). Rabbit liver growth hormone receptor and serum binding protein. Purification, characterization, and sequence. *J. Biol. Chem.* **263**, 7862–7867.
23. Karlsson, R., Michaelsson, A. & Mattson, A. (1991). Kinetic analysis of monoclonal antibody–antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods* **145**, 229–240.

Received: **10 Apr 1994**; revisions requested: **28 Apr 1994**; revisions received: **16 May 1994**. Accepted: **17 May 1994**.