Zinc Mediation of the Binding of Human Growth Hormone to the Human Prolactin Receptor

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Human growth hormone (hGH) elicits a diverse set of biological activities including lactation that derives from binding to the prolactin (PRL) receptor. The binding affinity of hGH for the extracellular binding domain of the hPRL receptor (hPRLbp) was increased about 8000-fold by addition of 50 micromolar $ZnCl_2$. Zinc was not required for binding of hGH to the hGH binding protein (hGHbp) or for binding of hPRL to the hPRLbp. Other divalent metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+}) at physiological concentrations did not support such strong binding. Scatchard analysis indicated a stoichiometry of one Zn^{2+} per hGH·hPRLbp complex. Mutational analysis showed that a cluster of three residues (His^{18}, His^{21}, and Glu^{174}) in hGH and His^{188} from the hPRLbp (conserved in all PRL receptors but not GH receptors) are probable Zn^{2+} ligands. This polypeptide hormone-receptor "zinc sandwich" provides a molecular mechanism to explain why nonprimate GHs are not lactogenic and offers a molecular link between zinc deficiency and its association with altered functions of hGH.

HUMAN GROWTH HORMONE CAUSES a variety of physiological and metabolic effects in various animal models, including linear bone growth, lactation, activation of macrophages, insulin-like and diabetogenic effects, and others (*1*). These effects derive from the interaction between hGH and specific cellular receptors. To begin to understand the molecular basis for the pleiotropic receptor binding properties of hGH, we have analyzed binding determinants between hGH and its cloned liver receptor (*2–4*). We have extended this approach to the cloned hPRL receptor (*5*), and found that Zn^{2+} is required for tight binding of hGH to the hPRL receptor but not for binding to the hGH receptor. Thus we show that a metal ion mediates a direct interaction between a polypeptide hormone and its extracellular receptor.

The extracellular domain of the hPRL receptor (hPRLbp) was expressed and secreted into the periplasm of *Escherichia coli* (Fig. 1A) in a manner similar to that designed for the extracellular binding domain of hGH receptor (hGHbp) (*6*). The hPRLbp was purified to near homogeneity from periplasmic extracts from *E. coli* by hGH affinity chromatography and gel filtration. The purified hPRLbp gave a single band of expected molecular weight (25 kD) on reduced SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1B). The purified hPRLbp had an amino-terminal sequence, (Q-L-P-P-G-K-P-E-I-F-K) (*7*), indicative of proper cleavage of the signal peptide.

Initially, the binding of hGH to the purified hPRLbp was weak and highly variable compared to binding to unpurified hPRLbp from *E. coli* periplasm. A series of dialysis experiments, treatments with chelating agents, and divalent metal ions showed that zinc was required for tight binding of hGH to the hPRLbp. Titration with $ZnCl_2$ at a fixed concentration of [^{125}I]hGH and hPRLbp established that formation of the hormone receptor complex was optimal at 50 μM $ZnCl_2$ (Fig. 2A).

Under these conditions binding of hGH to hPRLbp is about 8000-fold stronger in the presence of 50 μM $ZnCl_2$ compared to buffer containing 1 mM EDTA (Table 1).

Table 1. Zinc dependence for binding of hGH or hPRL to their purified binding proteins (bp). Dissociation constants (K_D) for binding to hGHbp (0.1 nM final) were measured in assay buffer [20 mM tris·HCl (pH 7.5), 0.1% w/v BSA], by competitive displacement of [^{125}I]hGH and precipitation of the complex using the anti-hGHbp, Mab 5 (*2–4, 6*). Binding to the hPRLbp (0.01 nM final) was measured in assay buffer containing 50 μM $ZnCl_2$ as described in Fig. 2A. In the presence of $ZnCl_2$, the dissociation constants were identical whether analyzed by competition with [^{125}I]hGH or [^{125}I]hPRL; we report only those values determined by displacement of [^{125}I]hGH. For binding to the hPRLbp in the presence of 1 mM EDTA and no added divalent metal ions, [^{125}I]hPRL was the only suitable tracer because its binding affinity was essentially unaffected by EDTA.

Receptor	K_D (± SD) (nM)	
	hGH	hPRL
hPRLbp		
+ $ZnCl_2$	0.033 (± 0.06)	2.6 (± 0.6)
+ EDTA	270 (± 90)	2.1 (± 0.7)
hGHbp		
+ $ZnCl_2$	1.6 (± 0.4)	>100,000
+ EDTA	0.42 (± 0.07)	>100,000

In contrast, the binding constant of hPRL to the hPRLbp is essentially the same under either condition and is close to that measured previously for the full-length recombinant hPRL receptor [2 to 3 nM (*5*)]. Moreover, binding of hGH to hPRLbp in the presence of $ZnCl_2$ is nearly 100-fold stronger than for hPRL and more than

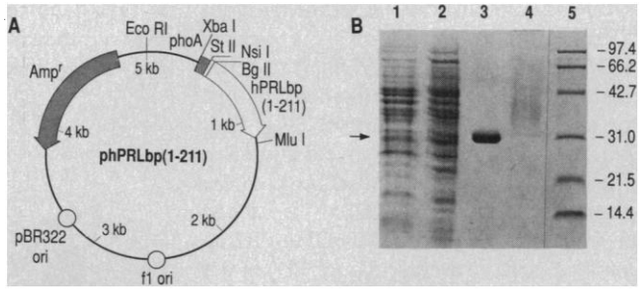

Fig. 1. (**A**) Diagram of plasmid phPRLbp(1–211) that directs secretion of the hPRLbp into the periplasm of *E. coli*. The hPRLbp gene fragment is transcribed under control of the alkaline phosphatase (phoA) promoter and secreted under direction of the St II signal sequence. Genes are indicated by arrows, replication origins by circles, and restriction sites used in the construction are indicated. A cDNA encoding the hPRL receptor (*5*) in a Bluescript plasmid (Stratagene) was purchased from P. Kelly (Royal Victorial Hospital, McGill University, Montreal, Canada). Site-directed mutagenesis (*25*) with an oligonucleotide with the sequence 5′-AGCCACAGA GATACGCGTCTATGTATCATTCAT-3′ was performed on this plasmid to introduce a stop codon and Mlu I restriction site (indicated by asterisks and underline, respectively) after the Thr^{211} codon that immediately precedes the transmembrane domain of the receptor. The 600-bp Bgl II–Mlu I fragment from this plasmid was then cloned into the Nsi I–Mlu I backbone of plasmid phGHbp(1–246) (*6*). A synthetic linker that spans the Nsi I and BglII sites was used to fuse the hPRLbp onto the St II secretion signal sequence and restore the 5′endof the hPRLbp gene. The bottom strand of this linker has the sequence 5′-GATCTCAGGTTTTCCAGGA GGTAACTGTGCA-3′. The top strand is complementary to this but 4 bp shorter on each end to match the restriction site termini. Dideoxy sequencing (*26*) was used to confirm the construction.(**B**) Coomassie-stained SDS-PAGE [12.5% (*27*)] of purified hPRLbp (indicated by arrow).The hPRLbp was purified essentially as described for the hGHbp (*6*), except that 50 μM $ZnCl_2$ was added to the ammonium sulfate precipitate prior to solubilizing and loading onto the hGH affinity column. The column was washed with 1 M KSCN and eluted with 2 M KSCN plus 50 mM NaCl, 0.02% NaN_3, 20 mM tris·HCl (pH 7.5). The eluate was dialyzed into the same buffer minus KSCN and stored frozen (at −70°C). Lanes 1 to 5 are an *E. coli* periplasmic fraction, the $(NH_4)_2SO_4$ precipipate, the protein after hGH affinity chromatography, the wash just before elution of hPRLbp, and molecular weight standards (ranging from 14 to 97 kD), respectively.

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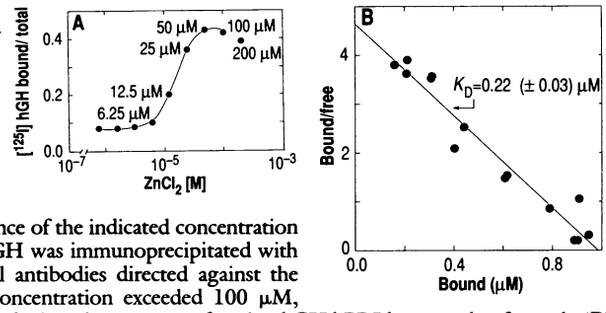

Fig. 2. (**A**) Binding of [^{125}I]hGH to the hPRLbp in the presence of 0.1% BSA (crystallized high-grade fraction V; Sigma), 140 mM NaCl, 10 mM $MgCl_2$, 20 mM tris (pH 7.5), and variable concentrations of total $ZnCl_2$. A fixed 1:1 ratio of hGH and hPRLbp (0.01 nM final) was incubated for 16 hours in the presence of the indicated concentration of $ZnCl_2$ and the bound [^{125}I]hGH was immunoprecipitated with affinity purified rabbit polyclonal antibodies directed against the hPRLbp (*28*). When the zinc concentration exceeded 100 μM, some protein precipitated thus reducing the amount of native hGH·hPRLbp complex formed. (**B**) Equilibrium dialysis for binding of $^{65}Zn^{2+}$ to the hGH·hPRLbp complex. All stock solutions were made from metal-free deionized water, and reagents were of highest quality available. Plastic dialysis cells and 3500 molecular weight cutoff membranes (preboiled in 5% w/w $NaHCO_3$ and washed) were soaked in 1 mM EDTA and washed thoroughly with dialysis buffer containing 20 mM tris·HCl (pH 7.5), 10 mM $MgCl_2$ (used to reduce nonspecific binding of Zn^{2+} to dialysis membrane), and 140 M NaCl. The hGH·hPRLbp complex (1.2 μM final) in 100 μl dialysis buffer was added to one half of the dialysis cell along with unlabeled $ZnCl_2$ diluted over a range from 10 μM to 0.1 μM. The $^{65}ZnCl_2$ tracer (21.84 μCi/μg, Dupont) was added in 100 μl of dialysis buffer to the other half of the dialysis cell. Cells were sealed and rotated slowly for 16 hours at 25°C. Aliquots (50 μl) from each half of the dialysis cell were counted, and bound and free zinc concentrations were calculated. The binding studies were performed in the absence of carrier protein to avoid adventitious binding of Zn^{2+}. This is a typical experiment but in two other experiments the K_D values were 0.4 and 0.6 μM.

10-fold stronger than the affinity of hGH for one hGHbp. Scatchard analysis shows a stoichiometry of one hormone to one hPRLbp, and the binding of hGH is competitive with hPRL in the presence or absence of zinc, indicating that the hormone binding sites on the hPRLbp overlap. In contrast, zinc actually lowers the affinity of hGH to hGHbp by four-fold, and prolactin does not bind to hGHbp in the presence or absence of $ZnCl_2$. Thus the two receptors have fundamentally different metal ion requirements in addition to their well-known hormone specificities.

Equilibrium dialysis experiments with $^{65}ZnCl_2$ were performed at a 1:1 ratio of hGH and hPRLbp (1.2 μM final) to determine the affinity and stoichiometry of zinc for the hGH·hPRLbp complex (Fig. 2B). Zinc binds in a noncooperative fashion to a single site in the hGH·hPRLbp complex (the maximal level of bound Zn^{2+} is 1.0 μM in the presence of 1.2 μM hGH·hPRLbp complex) with an average K_D (± SD) of 0.4 (± 0.2) μM from this plus two other independent experiments. Tight binding of zinc requires the presence of both hGH and hPRLbp (*8*), suggesting that the zinc site is at the interface of the complex. The higher affinity of Zn^{2+} for the hGH·hPRLbp complex in Fig. 2B compared to Fig. 2A reflects the absence of 0.1% bovine serum albumin (BSA) in the equilibrium dialysis experiment, which competes with the complex for binding of Zn^{2+}.

Scanning-mutational analysis has identified about a dozen side chains in hGH that mediate strongly the binding of hGH to hPRLbp in the presence of $ZnCl_2$ (*8*). Of these residues, only the side chains of His^{18}, His^{21}, and Glu^{174} are good candidate ligands for coordinating Zn^{2+}. These three residues are clustered (Fig. 3) when mapped upon a model of hGH (*3, 4*) built by homology to a folding diagram reported for porcine GH (*9*); His^{18} and His^{21} are on adjacent turns of helix 1 and are positioned near Glu^{174} on helix 4. All three face in the same direction and form a plausible site for binding of Zn^{2+}.

In the presence of zinc, replacing either His^{18}, His^{21}, or Glu^{174} with Ala reduces the hormone affinity for the hPRLbp by about 100-fold relative to wild-type (wt) hGH (Table 2). However, in 1 mM EDTA there is almost no difference in binding affinity between these mutants and wt hGH. Other Ala variants that disrupt binding of hGH to hPRLbp (*8*) produce the same reduction in binding whether in the presence of zinc or EDTA. The proximity of Asp^{171} to His^{18}, His^{21}, and Glu^{174} (Fig. 3) suggested that it could also be a zinc ligand but its mutation to Ala did not alter the binding of hGH and hPRLbp in the presence of zinc (Table 2).

In order to further evaluate the direct involvement of His^{18}, His^{21}, and Glu^{174} in binding zinc with the hPRLbp, we analyzed the binding of low concentrations of $^{65}Zn^{2+}$ to each hGH mutant by equilibrium dialysis (Table 3). The ratio of bound to free Zn^{2+} is dramatically reduced for these three mutants compared to wt hGH. The disruptive binding effects caused by Ala substitution of these residues correlate with disruption in zinc binding to the hGH·hPRLbp complex.

The model for the zinc binding site on hGH (Fig. 3) may account for the weak or undetectable binding of nonprimate growth hormones to prolactin receptors. Sequence alignments (*10*) of nonprimate growth hormones show that all 19 nonprimate GHs contain His^{21} and Glu^{174}, but instead of His^{18} they contain Gln^{18}. Interestingly, 17 of 19 nonprimate GHs contain His^{19} (hGH contains Arg^{19}). However, His^{19} cannot coordinate zinc along with His^{21} because they are on opposite sides of helix 1 (Fig. 3).

A natural variant of hGH, known as hGH-V, binds more tightly to somatogenic than lactogenic receptors (*11*). This homolog differs by only 13 residues out of 191 from hGH (*12*). Remarkably, instead of His^{18} and His^{21}, hGH-V contains Arg^{18} and Tyr^{21}. Our studies suggest that hGH-V will not bind Zn^{2+} in association with the hPRL receptor, and that this may be a major

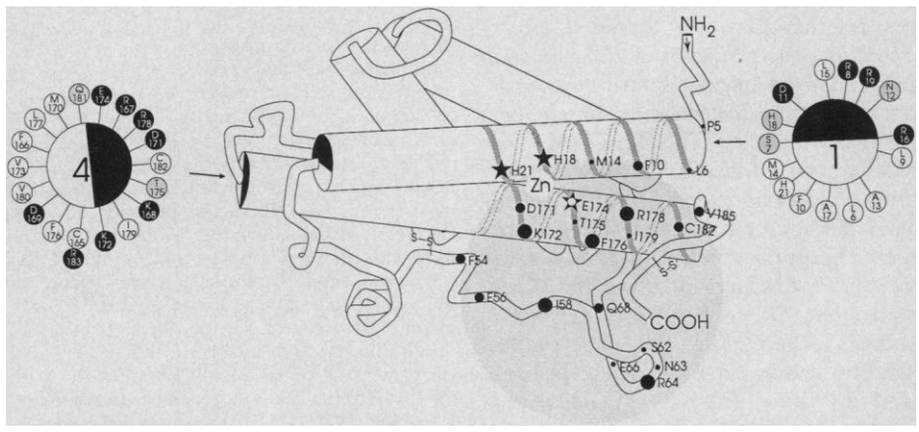

Fig. 3. Proposed Zn^{2+} binding site on hGH that mediates binding to the hPRLbp. The model of hGH [taken from (*29*)] is based upon the x-ray structure of pGH (*9*). Helical wheel projections show the amphipathic character of helix 1 and 4 with polar (shaded) and charged residues (blackened) on one face of the helix and nonpolar (open) on the other (*7*). The positions of the putative zinc binding ligands, His^{18}, His^{21}, and Glu^{174}, that are involved in binding hGH to the hPRLbp are shown (★). The region where hGH binds to the hGHbp is defined roughly by the shaded circle. Residues marked by the symbols, •, ●, ●, and ○ represent sites where Ala mutations in hGH cause reductions of two- to fourfold, four- to tenfold, greater than tenfold, or a fourfold increase in binding affinity for the hGHbp, respectively.

Table 2. Zinc dependence for binding of various Ala mutants (7) of hGH to the hPRLbp. Dissociation constants (K_D) between hGH and hPRLbp were determined as described in Fig. 2A. Mutants of hGH were produced and purified as previously described (*4*). Variants are designated by the wild-type (wt) residue followed by the position in hGH and the mutant residue; ND, not determined.

hGH mutant	+$ZnCl_2$ (50 μM)		+EDTA (1 mM)	
	K_D (± SD) (nM)	K_D(mut)/K_D(wt)	K_D (± SD) (nM)	K_D(mut)/K_D(wt)
wt	0.033 ± 0.006	1	270 ± 90	1
H18A	4.5 ± 1.1	135	370 ± 120	1.4
H21A	3.0 ± 1.0	91	200 ± 60	0.74
E174A	12.0 ± 3.0	356	360 ± 120	1.3
D171A	0.037 ± 0.011	1.1	ND	

Table 3. Ratio of bound to free $^{65}Zn^{2+}$ (0.2 μM total) in a fixed concentration of hGH mutant (7) and hPRLbp (each 2 μM). $^{65}ZnCl_2$ was allowed to equilibrate in dialysis cells, and bound and free zinc concentrations were determined as described in Fig. 2B.

hGH mutant	[$^{65}Zn^{2+}$] bound/free
wt	7.2 ± 0.2
H18A	0.1 ± 0.1
H21A	1.6 ± 0.1
E174A	1.0 ± 0.2

Table 4. Effect of mutations (7) at conserved His and Cys residues in hPRLbp on binding of hGH in the presence of $ZnCl_2$. Mutants of the hPRLbp were produced by site-directed mutagenesis (*25*) and were purified and assayed in the presence of Zn^{2+} as described in Figs. 1 and 2A and Table 1.

hPRLbp mutant	K_D (± SD) (nM)
wt	0.050 ± 0.008
H159A	0.047 ± 0.008
C184A	0.045 ± 0.004
H188A	>100

reason for its weaker binding.

The zinc independence for binding of hPRL to the hPRLbp is not as clear. All 13 prolactins aligned and numbered according to the sequence of hGH contain His^{21} and Asp^{174} (instead of Glu^{174}), and 10 of 13 contain His^{18}. The Asp^{174} may not bind Zn^{2+}, or alternatively hPRL may use a different set of residues to bind to the hPRLbp.

The zinc binding site is positioned on the edge of the epitope identified for binding to the hGH receptor (Fig. 3). The model suggests zinc reduces binding of hGH to the hGHbp (Table 1) by sterically interfering with binding of hGHbp. Binding to the hGHbp is enhanced about fourfold by mutation of Glu^{174} to Ala (*4*). Thus, while Glu^{174} hinders binding of hGH to hGHbp, it is required for zinc-mediated binding to hPRLbp.

Zinc typically coordinates four ligands in proteins (*13, 14*). Having identified three ligands from hGH and realizing that both hGH and the hPRLbp are required for tight zinc binding, we evaluated the possibility that the fourth ligand comes from the hPRLbp, the best candidates being His or Cys residues that are conserved in hPRLbp but absent in the hGHbp. An alignment of four GH (*2, 15*) and four PRL receptors sequences (*5, 16*) numbered according to the hPRLbp sequence (*5*) shows that His^{159}, Cys^{184}, and His^{188} are completely conserved in all PRL receptors but not GH receptors.

We mutated His^{159}, His^{188}, and Cys^{184} separately to Ala and expressed them in *E. coli*. Of these three hPRLbp mutants, only H188A reduced binding affinity (Table 4). Indeed, the binding affinity for hGH was reduced more than 2000-fold for the H188A mutant in the presence of zinc, which was below the limit of accurate measurement in the assay. The mutational analysis supports a model wherein zinc is bound by three ligands from hGH and one from hPRLbp.

The total concentration of zinc in serum varies from 5 to 20 μM in the adult population (*17*) and about 95% is complexed with proteins, mostly to serum albumin (*18*). Thus the free Zn^{2+} concentration in serum would be expected to range from about 0.25 to 1 μM. The K_D (0.4 ± 0.2 μM) for Zn^{2+} binding to the hGH·hPRLbp complex is in this range, suggesting that natural fluctuations in total zinc concentration can modulate the interaction between hGH and the hPRLbp complex.

It is nearly impossible to reconstitute serum so that the binding of specific metal ions can be tested independently because all metals compete to some extent for binding to serum proteins, notably serum albumin and metallothionene. Therefore, to translate our in vitro studies to a physiologically relevant setting is difficult. Nonetheless, in the presence of 0.1% (w/v) BSA (metal-free) the binding of hGH to the hPRLbp is modulated over a physiologically relevant range of total zinc (Fig. 2A). Under these conditions we evaluated the effect of other divalent metal ions (Table 5). At 60 pM hGH and hPRLbp, 20 μM Zn^{2+} supports about 50% complexation of hGH and hPRLbp, whereas no other metals at their maximal expected total serum concentrations do not promote substantial binding. At much higher concentrations of hGH and hPRLbp (5 nM), zinc supports 75% complexation. This represents the maximum amount of complex that can be precipitated in our assay with a polyclonal antibody and polyethylene glycol. Other metals have no effect, except for Cu^{2+} and Ca^{2+}, which support 29% complex formation. However, the K_D values (determined by Scatchard analysis) for binding of [^{125}I]hGH to hPRLbp in the presence of 5 mM $CaCl_2$ or 20 μM $CuSO_4$ are 21 (± 5 nM) and 11 (± 3) nM, respectively. These affinities are 300- to 600-fold weaker than for the zinc-mediated complex (0.03 nM, Table 1). Thus only zinc is capable of supporting strong binding between hGH and hPRLbp.

Interpretation of binding studies of hGH to receptors on cultured cells or tissues has often been problematic because usually these sources contain both prolactin and growth hormone (GH) receptors. Workers have traditionally used nonprimate GHs or prolactins to differentiate these receptors (*1, 2, 5*). Our data suggest that binding of hGH in the presence of Zn^{2+} or EDTA can readily distinguish these two receptor classes. For example, $ZnCl_2$ enhances binding of hGH to rat adipocytes (*19*) which suggests these cells contain prolactin-like receptors. In addition, studies analyzing binding of hGH to prolactin receptors should be controlled for the presence of zinc.

Zinc plays a central role in many endocrine functions [for review, see (*20*)] including growth hormone action. Zinc deficiency is often associated with alcoholism, pregnancy, some gastrointestinal disorders, severe burns, chronic renal failure, genetic disorders, and malnutrition. Moderate zinc deficiency leads to growth retardation (*20, 21*) and hyperprolactinemia (*22*). Our data provide a possible molecular basis for the association between zinc deficiency and altered growth hormone actions.

Zinc is a crucial component of the large class of zinc finger proteins (notably the steroid hormone receptors) that are important regulators of transcription [for reviews, see (*14*)]. Insulin is stored in complex with zinc in pancreatic cell secretory granules [for review, see (*23*)]. Our studies extend the

Table 5. Effect of divalent cations on the complexation of [^{125}I]hGH to hPRLbp (at 60 or 5000 pM) at physiological total serum concentrations (*17*). Highly purified metal ion salts for $CaCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$ were obtained from Johnson-Matthey Purotonic, Sigma, Mallinckrodt, Mallinckrodt and Johnson-Matthey Purotonic, respectively. Binding assays were performed as described in Fig. 2 in 0.1% BSA, 140 mM NaCl, 20 mM tris (pH 7.5) at 25°C. The percentage of complex formed was calculated from the ratio of the amount of [^{125}I]hGH·hPRLbp complex immunoprecipitated to total [^{125}I]hGH present in the assay.

Divalent metal (concentration)	Complex formation (%)	
	60 pM	5000 pM
None	3.8 ± 1.4	5.4 ± 0.8
Ca^{2+} (5 mM)	1.3 ± 0.7	29.0 ± 1.4
Co^{2+} (5 nM)	1.3 ± 2.4	6.8 ± 1.5
Cu^{2+} (20 μM)	1.7 ± 1.5	29.0 ± 1.4
Mg^{2+} (2 mM)	0.8 ± 1.4	9.1 ± 3.6
Mn^{2+} (2 μM)	0.4 ± 0.8	8.2 ± 1.8
Zn^{2+} (20 μM)	46.0 ± 3.0	74.0 ± 1.0

involvement of zinc in hormone action by showing that it mediates directly the interaction between a polypeptide hormone and an extracellular receptor. The growth hormone and prolactin receptors are homologous to receptors of the cytokine super family (*24*) which includes the interleukin-2 (IL-2), IL-3, IL-4, IL-6, IL-7, erythropoietin, and GM-CSF receptors. It is possible that zinc may mediate other hormone-receptor interactions such as these.

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7. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His, I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. Antibodies were prepared by immunizing rabbits with *E. coli*–derived hPRLbp by standard methods, and serum was collected after each boosting. Serum was passed over an hGH column (*6*) in which the hPRLbp was covalently cross-linked to the hGH by reaction with dimethylsuberimidate (10 mM final in phosphate-buffered saline; PBS). The hGH-hPRLbp column was washed sequentially with 2 M NaCl, 8 M urea, and 3 M KSCN to remove noncovalently bound components. Serum was passed over an hGH column to remove any antibodies to hGH that may have been produced from a low level contamination by hGH during hGH affinity purification by the hPRLbp immunogen. The flow-through was adsorbed onto the hGH-hPRLbp column, washed with 1 M NaCl, and eluted with 3 M KSCN. The nonblocking antibodies to hPRLbp were dialyzed into PBS and titered for the optimal concentration needed to precipitate hPRLbp in the assay.
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Stable, Monomeric Variants of λ Cro Obtained by Insertion of a Designed β-Hairpin Sequence

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λ Cro is a dimeric DNA binding protein. Random mutagenesis and a selection for Cro activity have been used to identify the contacts between Cro subunits that are crucial for maintenance of a stably folded structure. To obtain equivalent contacts in a monomeric system, a Cro variant was designed and constructed in which the antiparallel β-ribbon that forms the dimer interface was replaced by a β-hairpin. The engineered monomer has a folded structure similar to wild type, is significantly more stable than wild type, and exhibits novel half-operator binding activity.

ONE TEST OF OUR UNDERSTANDING of protein structure is the design of analogs of minimal complexity that retain the ability to fold as they would in the intact protein (*1*). Although dimers of λ Cro are composed of two distinct globular domains (*2*), contacts from both subunits are required for stability, since dissociation of the wild-type (wt) dimer is normally accompanied by denaturation (*3*). In this report we identify the intersubunit contacts that are required for Cro stability and describe the construction and characterization of an engineered Cro variant in which the connectivity of the polypeptide chain is altered to maintain these contacts in a stably folded monomer. This engineered monomer defines a simplified folding unit that retains the basic structural features of Cro.

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As shown in Fig. 1, A and B, the dimer interface of Cro consists of a β-strand from the carboxyl-terminus of each subunit, which pair to form an antiparallel β-ribbon (*2*). Residues 52 to 58 from each subunit form this structure and account for most of the intersubunit contacts. We probed the importance of the side chains at these positions by randomization using cassette mutagenesis (*4*) followed by a biological selection for Cro-mediated repression. The active sequences that were obtained are shown in Fig. 2A. Substantial sequence variability is allowed at every position except 58, where only the wt Phe and the conservative Tyr substitution are tolerated. These results suggest that the nature of the side chain at position 58 is critical for Cro function, whereas the identities of the side chains at positions 52 to 57 are less important. In the crystal structure (*2*), the Phe^{58} side chain (colored red in Fig. 1B) from one subunit packs into a crevice on the other subunit,