DeMarzo, A. M., Beck, C. A., Onate, S. A., & Edwards, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 72.
El-Ashry, D., Onate, S. A., Nordeen, S. K., & Edwards, D. P. (1989) *Mol. Endocrinol. 3*, 1545.
Gravanis, A., Schaison, G., George, M., de Brux, J., Satyaswaroop, P. G., Baulieu, E. E., & Robel, P. (1985) *J. Clin. Endocrinol. Metab. 60*, 156.
Guiochon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M., & Milgrom, E. (1988) *Nature 336*, 695.
Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., & Milgrom, E. (1989) *Cell 57*, 1147.
Hill, A. V. (1910) *J. Physiol. (London) 40*, iv.
Horwitz, K. B. (1985) *Endocrinology 116*, 2236.
Hurd, C., & Moudgil, V. K. (1988) *Biochemistry 27*, 3618.
Hurd, C., Nakao, M., Eliezer, N., & Moudgil, V. K. (1991) *Mol. Cell. Biochem. 105*, 73.
Koering, M. J., Healy, D. L., & Hodgen, G. D. (1986) *Fertil. Steril. 45*, 280.
Meyer, M.-E., Pornon, A., Ji, J., Bocquel, M.-T., Chambon, P., & Gronemeyer, H. (1990) *EMBO J. 9*, 3923.
Moguilewsky, M., & Philibert, D. (1985) in *The Antiprogestin Steroid RU486 and Human Fertility Control* (Baulieu, E. E., & Segal, S. J., Eds.) p 87, Plenum, New York.
Moudgil, V. K., & Hurd, C. (1987) *Biochemistry 26*, 4993.
Moudgil, V. K., Anter, M. J., & Hurd, C. (1989) *J. Biol. Chem. 264*, 2203.
Mullick, A., & Katzenellenbogen, B. S. (1986) *Biochem. Biophys. Res. Commun. 135*, 90.
Philibert, D. (1984) in *Adrenal Steroid Antagonism* (Agarwal, M. K., Ed.) p 77, deGruyter and Co., Berlin.
Philibert, D., Moguilewsky, M., Mary, I., Lecaque, D., Tournemine, C., Secchi, J., & Deraedt, R. (1985) in *The Antiprogestin Steroid RU486 and Human Fertility Control* (Baulieu, E. E., & Segal, S. J., Eds.) p 49, Plenum, New York.
Sasson, S., & Notides, A. C. (1988) *Mol. Endocrinol. 2*, 307.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Seaver, S. S., Van Eys, D. C., Hoffman, J. F., & Coulson, P. B. (1980) *Biochemistry 19*, 1410.
Skafar, D. F. (1991) *Biochemistry 30*, 6148.
Spelsberg, T. C., Boyd, P. A., & Halberg F. (1979a) *Adv. Exp. Med. Biol. 117*, 225.
Spelsberg, T. C., Thrall, C., Martin-Dani, G., Webster, R. A., & Boyd, P. A. (1979b) in *Ontogeny of Receptor and Reproductive Hormone Action* (Hamilton, T. H., & Clark, J. H., Eds.) p 31, Raven Press, New York.
Theofan, G. S., & Notides, A. C. (1984) *Endocrinology 114*, 1173.
Weichman, B., & Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856.

Selecting High-Affinity Binding Proteins by Monovalent Phage Display†

Henry B. Lowman, Steven H. Bass, Nancy Simpson, and James A. Wells*

Department of Protein Engineering, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

Received August 15, 1991; Revised Manuscript Received September 23, 1991

ABSTRACT: Variants of human growth hormone (hGH) with increased affinity and specificity for the hGH receptor were isolated using an improved phage display system. Nearly one million random mutants of hGH were generated at 12 sites previously shown to modulate binding to the hGH receptor or human prolactin (hPRL) receptor. The mutant hormones were displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. After three to six cycles of enrichment for hGH-phage particles that bound to hGH receptor beads, we isolated hGH mutants that exhibited consensus binding sequences for the hGH receptor. Residues previously identified as important for hGH receptor binding by alanine-scanning mutagenesis were more highly conserved by this selection method. However, other residues nearby were not optimal, and by mutating them, hormone variants having greater affinity and selectivity for the hGH receptor were isolated. This approach should be useful for those who wish to modify and understand the energetics of protein–ligand interfaces.

The design of variant hormones with enhanced receptor affinity and selectivity is important for dissecting the biological activities of hormones as well as creating new ones with greater therapeutic potential. Human growth hormone exhibits a variety of biological effects [for review see Isaksson et al. (1985)] that are initiated by binding to specific receptors, such as the hGH[1] receptor and hPRL receptor [for review see Hughes and Friesen (1987)]. Alanine-scanning mutational analysis (Cunningham & Wells, 1989, 1991) has identified sets of nonidentical but overlapping residues (epitopes) that modulate the binding of hGH to the extracellular domains of the hGH and hPRL receptor (called hGHbp and hPRLbp) (Figure 1). In one case, an alanine replacement at Glu174

† H.B.L. is supported by NIH Postdoctoral Grant GM13560-02.
* To whom correspondence should be addressed.

[1] Abbreviations: hGH, human growth hormone; hPRL, human prolactin; hGHbp, hGH binding protein consisting of the extracellular domain of the hGH receptor; hPRLbp, hPRL binding protein consisting of residues 1–211 of the hPRL receptor; Mab, monoclonal antibody; BSA, bovine serum albumin. Single mutants are designated by the wild-type residue (in single-letter code) followed by its position and the mutant residue. Multiple mutants are indicated by a series of single mutants separated by slashes; for example, E174S/F176Y denotes a double mutant in which Glu174 and Phe176 are converted to Ser and Tyr, respectively.

0006-2960/91/0430-10832$02.50/0

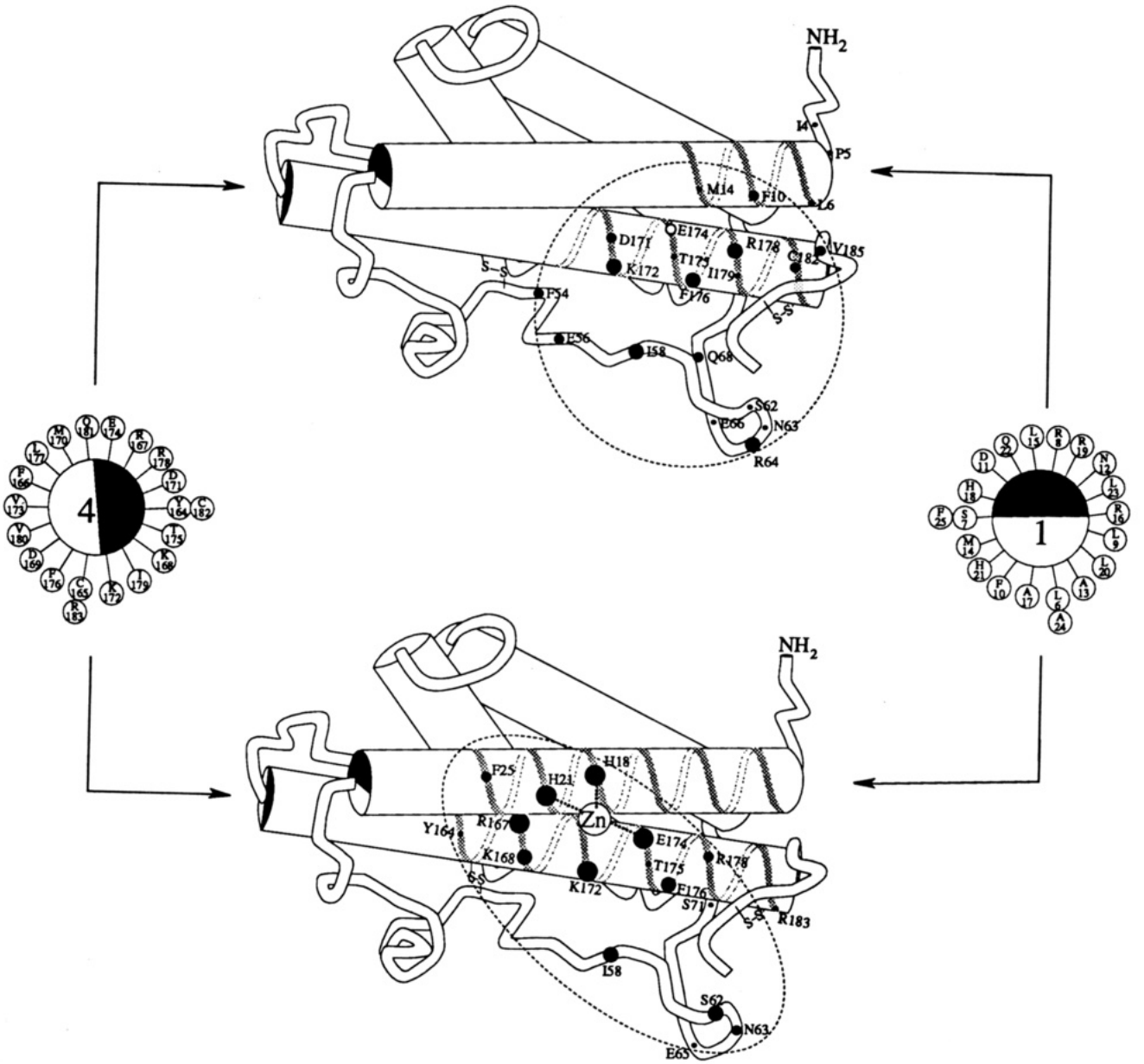

FIGURE 1: The regions of hGH implicated by mutagenesis studies (Cunningham et al., 1989, 1991) as important for binding to the extracellular domains of the hGH receptor (top) or hPRL receptor (bottom) are shown by the dashed enclosures on a cartoon based on the X-ray structure of pGH (Abdel-Meguid, 1987). Sites where alanine substitutions caused 2–4-fold, 4–10-fold, 10–80-fold, or >80-fold reductions in binding affinity for each receptor are shown by ·, •, ●, and ⬤, respectively; the E174A mutation (○) caused a 4-fold increase in binding affinity to the hGHbp. A zinc ion (Zn) is crucial for binding hGH to the hPRLbp (bottom) but not to the hGHbp (top).

enhanced binding affinity to the hGHbp and reduced binding to the hPRLbp. This raised the questions, what is the optimal binding sequence(s) for the hGHbp and how do we go about finding it among the myriad of possibilities?

In theory, the method of phage display (Smith, 1985; Parmley & Smith, 1988) offers the possibility to select in vitro (by affinity chromatography) among a population of variant proteins for high-affinity mutants. To test this method, peptides (Scott & Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990) and even functional proteins (Bass et al., 1990; McCafferty et al., 1990) have been fused to the gene III pili attachment protein of filamentous phage. These "fusion phage" were shown to bind immobilized protein ligands in vitro. For example, successive rounds of binding enrichment to various immobilized Mabs allowed sorting of peptide libraries displayed in a polyvalent fashion (3–5 copies per phage) to isolate peptide sequences with moderate binding affinity (K_D $\sim 10^{-5}$–10^{-7}; Cwirla et al., 1990). Multipoint attachment of the polyvalent-displayed peptides (a "chelate" effect) was thought to limit the ability to obtain higher affinity peptides (Cwirla et al., 1990). However, by reducing the number of displayed copies of the fusion protein to one, we have shown (Bass et al., 1990) that it is possible to separate two mutants of hGH having variably high affinity for the hGHbp (K_D $\sim 10^{-8}$–10^{-10}).

Here, we describe improvements in the monovalent phage display system for hGH and show it is suitable for sorting libraries of hGH mutants for high-affinity binders (K_D $\sim$ 10^{-8}–10^{-11}). After sorting nearly one million hGH variants, we find that the ones with highest binding affinity contain substitutions near those residues previously shown to strongly modulate binding to the hGHbp.

Materials and Methods

Plasmid and Mutant Constructions. The plasmid phGHam-g3 (Figure 2) was constructed by site-directed

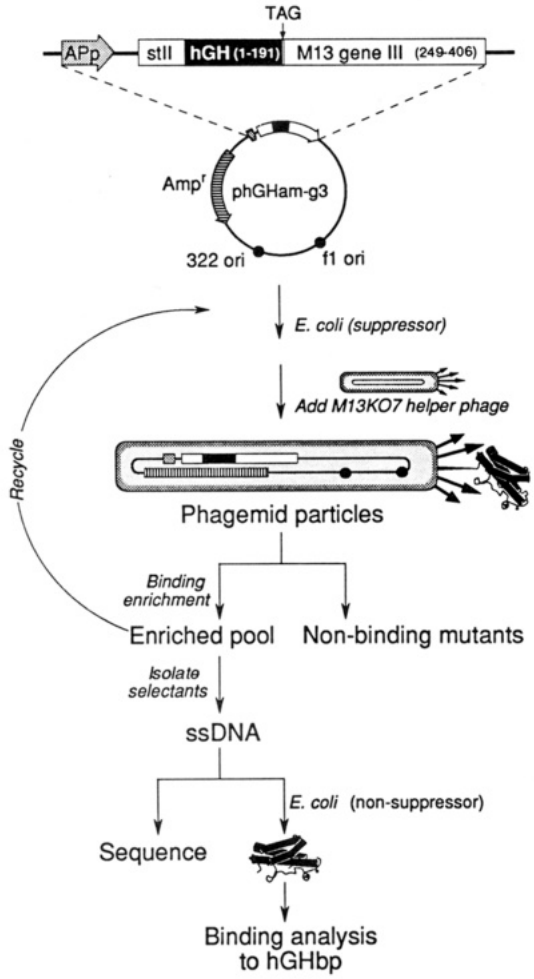

FIGURE 2: Strategy for using monovalent phage display for selection of mutants of hGH that bind hGHbp. The plasmid phGHam-g3 contains the alkaline phoshatase promoter (APp), the stII signal sequence, and the hGH gene followed by codons 249–406 of the M13 gene III. After several cycles of binding selection, individual phage are isolated and ssDNA sequenced to determine what amino acid substitutions are present. Finally, the ssDNA is transformed into a nonsuppressor *E. coli* strain, in which the amber codon (TAG) terminates translation and free hormone is produced. See text for details.

mutagenesis (Kunkel et al., 1987) of pS0132 (Bass et al., 1990), so that the hGH gene (codons 1–191) is followed by an amber triplet (TAG). This codon is suppressed as glutamine in the *sup*E *Escherichia coli* strain XL1-Blue (Bullock et al., 1987), when hGH phagemid particles are produced. The construction places hGH at the end of a region rich in Gly-Gly-Gly-Ser repeats that precedes the carboxyl-terminal domain (codons 249–406) of M13 gene III. To produce phagemid particles, male XL-1-Blue cells containing the phGHam-g3 (or mutated derivatives) were infected with M13K07 helper phage (Vierra & Messing, 1987) at a multiplicity of infection of approximately 100 helper phage per cell. Phagemid particles were prepared and propagated as described (Bass et al., 1990).

Mutagenic cassettes were inserted at various positions in the hGH gene of phGHam-g3 to obtain three separate random mutant libraries. Mutant libraries were created by substituting four residues simultaneously with the nucleotide sequence NNS (N = A/G/C/T; S = G/C) to generate all possible amino acid substitutions while limiting the total number of codons to 32. Four NNS codons yield 1.05×10^6 possible nucleotide sequences that encode 1.60×10^5 possible polypeptide chains. Only the amber stop triplet (TAG) is possible in the library, and it is suppressible in *sup*E strains of *E. coli*. For cassette mutagenesis (Wells et al., 1985) of helix 4, the unique *Bgl*II site (at hGH codon 137) of phGHam-g3 was destroyed, a new *Bgl*II site was introduced at hGH codon 183, and a unique *Bst*EII site was introduced at codon 161, followed by a TGA stop triplet and a single-base frame shift (called pH0509B). A cassette which mutated residues 172, 174, 176, and 178 (helix 4a) contained the complementary oligonucleotides 5′-pGT TAC TCT ACT GCT TTC AGG AAG GAC ATG GAC NNS GTC NNS ACA NNS CTG NNS ATC GTG CAG TGC A-3′ and 5′-pGA TCT GCA CTG CAC GAT SNN CAG SNN TGT SNN GAC SNN GTC CAT GTC CTT CCT GAA GCA GTA GA-3′. This was ligated with the large *Bst*EII–*Bgl*II fragment of pH0509B.

For mutagenesis of helix 1, a unique *Xho*I site followed by a single-base frame shift was introduced at codon 6, and a unique *Kpn*I site was introduced at codon 27 of hGH in phGHam-g3 to create pH0508B. The helix 1 library, which mutated hGH residues, 10, 14, 18, and 21, was constructed by ligating to the large *Xho*I–*Kpn*I fragment of pH0508B, a cassette made from the complementary oligonucleotides 5′-pTCG AGG CTC NNS GAC AAC GCG NNS CTG CGT GCT NNS CGT CTT NNS CAG CTG GCC TTT GAC ACG TAC-3′ and 5′-pGT GTC AAA GGC CAG CTG SNN AAG ACG SNN AGC ACG CAG SNN CGC GTT GTC SNN GAG CC-3′.

The helix 4b library, which mutated residues 167, 171, 175, and 179 within the E174S/F176Y background, was constructed using pH0509B as described above using the complementary oligonucleotides 5′-pG TTA CTC TAC TGC TCC NNS AAG GAC ATG NNS AAG GTC AGC NNS TAC CTG CGC NNS GTG CAG TGC A-3′ and 5′-pGA TCT GCA CTG CAC SNN GCG CAG GTA SNN GCT GAC CTT SNN CAT GTC CTT SNN GAA GCA GTA GA-3′. The *Bst*EII site was eliminated after ligation of the cassette. The cassette ligation products were extracted with phenol–chloroform, ethanol precipitated, resuspended in water, and electroporated (Dower et al., 1988) into XL1-Blue cells. From 58 clones, 17% contained no cassette insert, 17% were frame-shifted, and 7% had spurious mutations within the cassette at positions other than those targeted for NNS mutagenesis. On the basis of the sequences of 192 NNS codons from an unselected helix 4a library, the occurrences of nucleotides were 31% A, 18% T, 26% C, and 25% G at the first two positions. At the third position, the occurrences were 50% C, 49% G, and 1% A.

Binding Enrichments. Phagemid particles displaying mutants of hGH were cycled through rounds of binding selection to isolate variants that bind to hGHbp which was linked to oxirane–polyacrylamide beads as described (Bass et al., 1990). Competitive binding experiments with ^{125}I-hGH indicated that 58 fmol of functional hGHbp was coupled per microliter of bead suspension (about 50 μg dry bead weight). Although one hGH molecule can bind two hGHbp simultaneously (Cunningham et al., 1991b), the sequential order of binding and the affinity for the second hGHbp practically limit its binding to the single site shown (Figure 1) at this concentration of hGHbp.

After two to six cycles of binding selection (depending upon the library), individual phage were isolated and sequenced (Sanger et al., 1977). Because of the restriction sites introduced into the hGH gene for cassette mutagenesis, we were able to distinguish contaminating wild-type hGH from any wild-type hGH sequences that might have been selected from the cassette libraries. No wild-type sequences were selected that derived from the cassette mutagenesis. However, con-

taminating wild-type phGHam-g3 was found in the selected helix 4a library (14% of sequenced clones) and in the selected helix 4b library (22%) after several rounds of enrichment. These spurious phagemids likely resulted from equipment or airborne contamination.

Expression of Free hGH Variants. Variants of hGH were expressed as free hormones by transforming ssDNA from phagemid clones into strain 16C9, a nonsuppressor strain of *E. coli* (Chang et al., 1987). The variant hGH proteins were purified from 30-mL shake flasks (Cunningham & Wells, 1989; Olson et al., 1981). The concentration of protein was determined by laser densitometry of Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels using wild-type hGH as the standard, and binding to the hGHbp was determined in solution (Cunningham & Wells, 1989).

Results

Improvements in the Phage Display System. We have made three additional improvements to the phage display system for hGH (Figure 2). First, the fusion junction of hGH to gene III has been moved closer to the carboxyl terminus of gene III by deleting all but the last of several Gly-Gly-Gly-Ser repeats that are found in a region we presumed to be a flexible linker separating the amino and carboxyl domains of gene III. We believe this fusion junction at codon 249 of the gene III makes the target protein more accessible for binding when assembled into the phagemid particle. For example, phagemid particles produced from phGHam-g3 (Figure 2) and an earlier fusion, pS0132 (Bass et al., 1990), were tested with three antibodies to hGH (Medix 2, 1B5.G2, and 5B7.C10) that are known to have binding determinants near the carboxyl terminus of hGH (Cunningham et al., 1989; L. Jin and J. Wells, unpublished results) and one control antibody (Medix 1) that recognizes determinants in helices 1 and 3 away from the carboxyl terminus. Binding of pS0132 particles was reduced by >2000-fold for both Medix 2 and 5B7.C10 and reduced by >25-fold for 1B5.G2 compared to binding to the Medix 1 control. On the other hand, binding of phGHam-g3 phage was weaker by only about 1.5-fold, 1.2-fold, and 2.3-fold for the Medix 2, 1B5.G2, and 5B7.C10 antibodies, respectively, compared with binding to Medix 1. Accessibility to the carboxyl terminus is important for display of hGH because there are receptor binding determinants near the carboxyl terminus (Figure 1).

Second, an amber codon (TAG) was placed after the last codon of hGH to facilitate expression of the free hormone variant in a nonsuppressor strain of *E. coli* following binding selection (Figure 1). This avoids the need to construct chain-terminated variants of hGH. Finally, the alkaline phosphatase promoter was used because low-level expression can be maintained in high-phosphate-rich media when the monovalent hGH-phagemid particles are produced and high-level expression of the free hormone variant can be induced in a nonsuppressor strain by growth in low-phosphate media without the need to add an external inducer. Thus, the library construction, binding selection, sequencing, and expression are all directed from the same phagemid vector.

Binding Selection of High-Affinity hGH Mutants. We mutated residues in helix 1 and helix 4 of hGH that were shown by alanine-scanning mutagenesis to modulate the binding of the hGHbp and hPRLbp (Figure 1). Three libraries were constructed by cassette mutagenesis that fully mutated four residues at a time. Each library, encoding 20^4 possible variant hGH sequences, contained at least 10^7 independent transformants. We checked the starting libraries for randomness by sequencing 10–50 clones and/or by restriction enzyme analysis of the phagemid pools prior to binding selection. These studies confirmed that cassettes had been inserted in over 80% of the clones and there appeared to be no large bias for the nucleotides inserted.

We simultaneously randomized codons for K172, E174, F176, and R178 (Figure 1) in the helix 4a library (Table I). From additivity principles [for review see Wells (1990)], we presumed that most mutants would be dramatically reduced in binding affinity to the hGHbp because from the product

Table I: Identity of hGH Mutants from Three Separate Libraries after Various Cycles of Binding Selection to Immobilized hGHbp[a]

helix 4a library, hGH elution				helix 4a library, Gly, pH 2, elution			
K172	E174	F176	R178	K172	E174	F176	R178
One Cycle							
M	F	W	R	K	S	Y	R
S	H	V	R	R	S	Y	R
V	G	D	R	S	L	F	H
K	P	S	Q	Q	P	G	G
N	G	T	C	K	T	G	N
F	C	P	V	G	N	N	E
Three Cycles							
R	S	Y	R	K	A	Y	R (3)
R	F	F	R (2)	R	S	Y	R
K	T	Y	K	R	S	F	S
K	S	Y	T	R	G	Y	R
K	R	Y	R	R	S	F	R
R	T	Y	H	K	Q	Y	R
Q	W	F	R	K	T	Y	K
I	A	M	R				
Consensus							
		172	174	176	178		
		K	A	Y	R		
		R	S	F	K		
			T				

helix 1 library, Gly, pH 2, elution				helix 4b library, Gly, pH 2, elution			
F10	M14	H18	H21	R167	D171	T175	I179
Four Cycles							
H	G	N	N	N	S	T	T
A	W	D	N (2)	K	S	T	T
Y	T	V	N	S	N	T	T
I	N	I	N	D	S	T	T
L	N	S	H	D	S	T	T (+)
F	S	F	G	D	S	A	T
				D	S	A	N
				T	D	T	T
				N	D	T	N
				A	N	T	N
				A	S	T	T
Six cycles							
H	G	N	N (6)	N	S	T	T (2)
F	S	F	L	N	N	T	T
				N	S	T	Q
				D	S	S	T
				E	S	T	I
				K	S	T	L
Consensus							
H	G	N	N	N	S	T	T
				D	N		

[a] Half the mutants in the helix 4a library were sequenced after elution with hGH (0.4 μM) buffer (Bass et al., 1990) and the rest after subsequent elution with glycine (0.2 M, pH 2) buffer. All the mutants in the helix 1 and 4b libraries were sequenced after being washed with hGH buffer and eluted with glycine buffer. Numbers in parentheses indicate the number of times each mutant appeared among the selected cloned. One helix 4b mutant (+) contained a spurious R178H substitution. The consensus sequence after at least three cycles reflects those residues selected at each position which were significantly enriched (usually 5–10-fold) above their expected random frequency in the NNS library. See Materials and Methods for further details.

Table II: Binding of Purified hGH Mutants to hGHbp[a]

helix 4a sequence position					
172	174	176	178	K_D (nM)	K_D(mut)/ K_D(wt hGH)
K	S	Y	R	0.06 ± 0.01	0.18
R	S	F	R	0.10 ± 0.05	0.30
K	T	Y	K	0.16 ± 0.04	0.47
R	S	Y	R	0.20 ± 0.07	0.58
K	A	Y	R	0.22 ± 0.03	0.66
R	F	F	R	0.26 ± 0.05	0.76
K	Q	Y	R	0.33 ± 0.03	1.0
K	**E**	**F**	**R**	**0.34 ± 0.05**	**(1)**
R	T	Y	H	0.68 ± 0.17	2.0
R	S	F	S	1.10 ± 0.2	3.3
helix 1 sequence position					
10	14	18	21	K_D (nM)	K_D(mut)/K_D(wt
A	W	D	N	0.10 ± 0.03	0.30
H	G	N	N	0.14 ± 0.04	0.42
F	**M**	**H**	**H**	**0.34 ± 0.05**	**(1)**
F	S	F	L	0.68 ± 0.19	2.0
Y	T	V	N	0.75 ± 0.19	2.2
L	N	S	H	0.82 ± 0.20	2.4
I	N	I	N	1.20 ± 0.31	3.4
helix 4b sequence position					
167*	171*	175*	179*	K_D (nM)	K_D(mut)/ K_D(wt hGH)
N	S	T	T	0.04 ± 0.02	0.12
E	S	T	I	0.04 ± 0.02	0.12
K	S	T	L	0.05 ± 0.03	0.16
N	N	T	T	0.06 ± 0.03	0.17
R	**D**	**T**	**I**	**0.06 ± 0.01**	**(0.18)**
N	S	T	Q	0.26 ± 0.11	0.77

[a] See Figure 1 and Table I. Competition binding experiments were performed using ^{125}I-hGH, hGHbp, and the anti-receptor antibody Mab263 for immunoprecipitation of the complex. Note that all helix 4b mutations (*) are in the background containing the double mutant E174S/F176Y. See Materials and Methods for further details. The wild-type sequence is shown in boldface.

of the single alanine mutant effects (Cunningham & Wells, 1991) a tetraalanine mutant would be reduced by more than 3000-fold. Nonetheless, after only three cycles of binding selections on hGHbp beads (Figure 2) a strong consensus sequence developed (among 15 selected clones). For example, positions 172 and 178 usually contained a positively charged residue (Arg or Lys); position 174 usually contained Ala, Ser, or Thr; and position 176 contained a Phe or Tyr (Table I). This consensus was clear after three cycles and stronger after the glycine, pH 2, elution as compared to the hGH elution that preceded it. In subsequent libraries we selected clones that were eluted with glycine (pH 2) after an hGH wash, because this was the more stringent selection. Representative isolates were expressed as free hGH variants and analyzed for binding in vitro (Table II). These mutants bind in a narrow range of affinity, from 5-fold tighter (E174S/F176Y) to 3-fold weaker than wild-type hGH.

Four residues in helix 1 (F10, M14, H18, and H21) were similarly mutated, and after four and six cycles a non-wild-type consensus developed (Table I). Position 10 on the hydrophobic face of helix 1 (Figure 1) tended to be hydrophobic whereas positions 21 and 18 on the hydrophilic face were dominated by Asn. No consensus was evident for position 14. Among the selected clones free hormone variants bound to the hGHbp with an affinity that ranged from 3-fold tighter to 3-fold weaker than wild-type hGH.

The helix 4b library was constructed to further improve the binding affinity of the higher affinity double mutant (E174S/F176Y) selected from the helix 4a library. Residues surrounding positions 174 and 176, on the hydrophilic face of helix 4 (R167, D171, T175, and I179; Figure 1), were mutated in the E174S/F176Y double mutant. After six cycles of binding enrichment, a reasonably clear consensus sequence developed (Table I). Interestingly, all positions tended to contain polar residues, notably Ser, Thr, and Asn. Binding affinity of free hormone variants for the hGHbp ranged from 1.5-fold tighter to 4-fold weaker than the E174S/F176Y double mutant (Table II). Two multiple mutants (R167N/D171S/E174S/F176Y/I179T and R167E/D171S/E174S/F176Y) bound about 8-fold stronger to the hGHbp than wild type.

Discussion

Our data show that phage display can be useful to isolate high-affinity mutants of hGH for its receptor. For example, over half of the free hormone variants selected had binding affinities greater than wild-type hGH (up to 8-fold tighter). This suggests that there are a number of solutions to tight binding. In some cases hGH variants with lower affinities were isolated more frequently than higher affinity variants (e.g., K172R/E174F, K_D = 0.26 nM, versus E174S/F176Y, K_D = 0.06 nM; Table I). This could result from statistical fluctuation, differential effects of on- and off-rates (binding selections may be on-rate limited), or differences in expression levels of the mutants.

There are some nonadditive effects in binding that we see when residues are near each other. For example, the individual E174S and F176Y mutants cause a 3-fold enhancement and 9-fold reduction in binding affinity, respectively (Cunningham et al., 1990; Cunningham and Wells, unpublished results). If these mutants were purely additive, the double mutant would bind 3-fold weaker; yet it binds 5-fold tighter. Such nonadditive effects for neighboring substitutions are not unusual [for review see Wells (1990)]. However, this illustrates the power of phage binding selection as a means of selecting optimized mutants from a library randomized at several positions.

Several of the tighter hGHbp binding mutants that alter residues in the hPRLbp epitope (Figure 1) were tested for their ability to bind to the hPRLbp (not shown). Wild-type hGH binds tightly to the hPRLbp in the presence of $ZnCl_2$ (K_D = 33 pM; Cunningham et al., 1990). However, the E174S/F176Y mutant binds 200-fold weaker to the hPRLbp than hGH, probably because Glu174 is reqiured for coordination of Zn^{2+} in the complex (Figure 1). Since this mutant binds 5.6-fold tighter to the hGHbp than wild-type hGH, the change in preference for binding hGHbp over hPRLbp is about 1100-fold. The E174T/F176Y/R178K and R167N/D171S/E174S/F176Y/I179T mutants each bind >500-fold weaker to the hPRLbp than hGH, yielding changes in binding preference of >1000-fold and >4000-fold, respectively.

The disruptive effect of alanine substitutions at each of the 12 positions mutated for binding to the hGHbp (Figure 1) correlates well with their frequency of conservation after binding selection to the hGHbp in vitro (Figure 3A). This correlation is evident despite the fact that conservation was assessed in the background of other mutations, across three different libraries, and over a range of binding selections (three to six cycles). Thus, in our in vitro system functional importance correlates with sequence conservation. In contrast, studies of natural variants that may ascribe functional significance to sequence conservation can be grossly confounded because the protein ligand coevolves with its receptor, and/or the protein contains overlapping functional epitopes that evolve at different rates in vivo. In fact, this appears to be the case for this very epitope on hGH. The sequences of over 21 natural species variants of growth hormone are known (T. Bewley, personal communication). However, we find that the degree

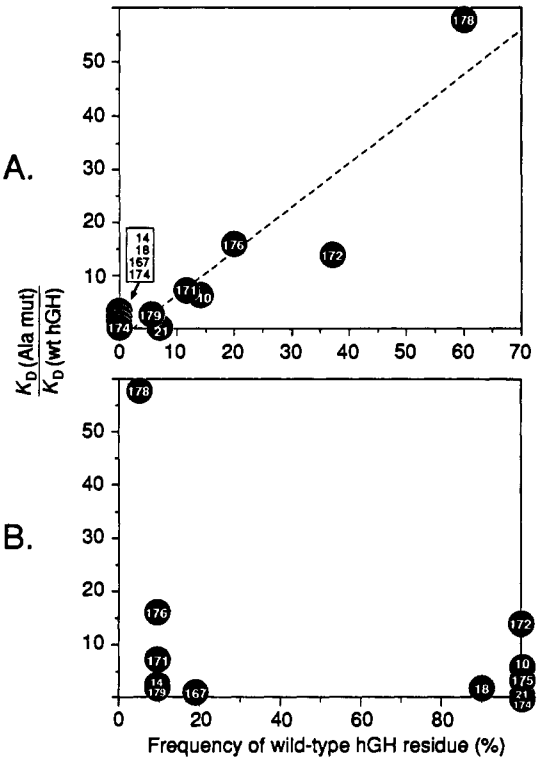

FIGURE 3: Panel A: Correlation between occurrences of wild-type residues in selected libraries and effects of single alanine substitutions on binding affinity to hGHbp. The effect of an alanine substitution (y-axis) at each position (Cunningham & Wells, 1991) is shown as a function of the percentage of clones (x-axis) from each selected library which contained the wild-type residue at that position (Table I). The T175 mutant has been omitted from the data set because data were not available for the alanine substitution, but only for T175S (Cunningham et al., 1990). A linear least-squares fit to the data ($y = -2.0 + 0.83x$) gives a correlation coefficient (R^2) of 0.86. Panel B: A similar plot where a set of 21 different natural growth hormone variants were used instead of those selected in vitro.

of sequence conservation is totally uncorrelated with the alanine-scanning functional studies (Figure 3B). Not surprisingly, 19 of these 21 growth hormones do not bind tightly to the hGHbp. Moreover, three of the residues in the family that are fully conserved (H18, H21, and E174) are irrelevant to hGHbp binding. These are crucial for zinc binding and storage of a dimeric form of growth hormone in vivo (Cunningham et al., 1991a), properties that are not important for binding to the hGH receptor. Thus, we suggest that the in vitro phage display system is much more controlled for studying molecular evolution of a single molecular interface.

We never recovered the wild-type sequence from any of the libraries except as a contaminant (Table I). This may have resulted from three effects. First, Ala substitutions at position 174 cause a 4-fold enhancement in binding, and this position gives a non-wild-type consensus residue. Second, two residues (H18 and R167) which showed very poor or non-wild-type consensus are not important for binding to the hGHbp (Figure 1). Thus there was less "selective pressure" for binding to the hGHbp beads. Finally, residues identified as being important by alanine scanning are not necessarily precluded from having isofunctional replacements.

Limitations in transformation efficiency practically restrict one to fully randomizing no more than six residues simultaneously. This problem can be mitigated using a combined strategy of alanine scanning to identify side chains that modulate binding and phage selection to randomly mutate these side chains and their neighbors. The most promising sites to mutate for enhancing affinity appear not to be those crucial for binding as they are most likely to be conserved after selection (Figure 3A); it is better to target residues nearby. For example, within the helix 4a library K172, F176, and R178 were largely conserved after binding selection, and alanine substitutions at each of these caused 10–50-fold reductions in binding affinity (Figure 1). In contrast, E174A enhanced binding affinity and E174 was never recovered after selection. Finally, we envision that combining optimized binding segments (much like shuffling exons) will produce even higher high-affinity and receptor-specific hormone analogues. In fact, preliminary experiments show that combining the highest affinity mutants from the helix 1 and a 4a libraries produces a multiple mutant that binds about 30-fold tighter to the hGHbp (K_D ~13 pM; H. Lowman and J. Wells, unpublished results).

ACKNOWLEDGMENTS

We thank Peter Ng and Mark Vasser for oligonucleotide synthesis, Ellson Chen for helping with DNA sequencing, Marjorie Winkler for providing hGHbp, and Rebecca Ward for critical reading and suggestions on the manuscript.

REFERENCES

Abdel-Meguid, S. S., Shieh, H. S., Smith, W. N., Dayringer, H. E., Violand, B. N., & Bentle, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6434–6437.

Bass, S., Greene, R., & Wells, J. A. (1990) *Proteins: Struct., Funct., Genet. 8*, 309–314.

Bullock, W. O., Fernandez, J. M., & Short, J. M. (1987) *Biotechniques 5*, 376–379.

Chang, C. N., Rey, M., Bochner, B., Heyneker, H., & Gray, G. (1987) *Gene 55*, 189–196.

Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081–1085.

Cunningham, B. C., & Wells, J. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3407–3411.

Cunningham, B. C., Jhurani, P., Ng, P., & Wells, J. A. (1989) *Science 243*, 1330.

Cunningham, B. C., Henner, D. J., & Wells, J. A. (1990) *Science 247*, 1461–1465.

Cunningham, B. C., Mulkerrin, M. G., & Wells, J. A. (1991a) *Science 253*, 545–548.

Cunningham, B. C., Ultsch, M., DeVos, A. M., Mulkerrin, M. G., Clauser, K. R., & Wells, J. A. (1991b) *Science* (in press).

Cwirla, S. E., Peters, E. A., Barrett, R. W., & Dower, W. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6378–6382.

Devlin, J. J., Panganiban, L. C., & Devlin, P. E. (1990) *Science 249*, 404–406.

Dower, W. J., Miller, J. F., & Ragsdale, C. W. (1988) *Nucleic Acids Res. 16*, 6127.

Hughes, J. P., & Friesen, H. G. (1987) *Annu. Rev. Physiol. 47*, 469–482.

Isaksson, O. G. P., Eclen, S., & Jansson, J. O. (1985) *Annu. Rev. Physiol. 47*, 483–499.

Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J., & Lerner, R. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4363–4366.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

McCafferty, J., Griffiths, A. D., Winter, G., & Chiswell, D. J. (1990) *Nature 348*, 552–554.

Olson, K. C., Fenno, J., Lin, N., Harkins, R. N., Snider, C., Kohr, W. H., Ross, M. J., Fodge, D., Prender, G., & Stebbing, N. (1981) *Nature 293*, 408–411.

Parmley, S. F., & Smith, G. P. (1988) *Gene 73*, 305–318.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Scott, J. K., & Smith, G. P. (1990) *Science 249*, 386-390.
Smith, G. P. (1985) *Science 228*, 1315-1317.
Vierra, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3-11.
Wells, J. A. (1990) *Biochemistry 29*, 8509-8517.
Wells, J. A., Vasser, M., & Powers, D. B. (1985) *Gene 34*, 315-323.

Articles

Marked Effects of Salt on Estrogen Receptor Binding to DNA: Biologically Relevant Discrimination between DNA Sequences†

Fern E. Murdoch, Kurt A. A. Grunwald, and Jack Gorski*

Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received May 31, 1991; Revised Manuscript Received August 26, 1991

ABSTRACT: Avidin-biotin complexed with DNA (ABCD) assays were employed to determine the binding affinity of estrogen receptor (ER) to DNA under various salt conditions. Type and concentration of salt in the reaction buffer dramatically affected the ability of the ER to discriminate between DNA sequences. Under appropriate salt conditions, ER was able to bind to the estrogen response element from the *Xenopus* vitellogenin A2 gene with at least 3 orders of magnitude greater affinity than a two base pair mutant sequence, and 5 orders of magnitude greater affinity than plasmid DNA. In these studies, the best discrimination was observed under conditions of salt type and concentration that more closely approximated intracellular conditions, i.e., 100-150 mM potassium salts. Analysis of the binding affinities for ER to all three types of DNA over a range of KCl concentrations indicated that the ionic interactions upon ER binding were the same for the three DNA molecules tested. Therefore, the additional stability of ER binding to target DNA sequences was contributed by nonionic interactions.

Estrogens bind with high affinity to a specific receptor protein localized in the cell nucleus. The estrogen receptor (ER)[1] contains a DNA binding domain capable of binding to specific sequences within a target gene termed estrogen response elements (ERE). This model for ER action predicts that the ER is capable of distinguishing an ERE from the mass of DNA sequences in the eukaryotic nucleus. Given the concentrations of ER and DNA in the mammalian nucleus, theoretical calculations predict a difference of 3-4 orders of magnitude in the binding affinities between target and nontarget DNA sequences is needed for the ER to find its ERE amidst the mass of DNA in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989). Recent studies of ER-DNA interactions have focused on target sequences, particularly the ERE derived from the *Xenopus* vitellogenin A2 gene (VitERE; Klein-Hitpass et al., 1986, 1988; Walker et al., 1984), with only an occasional comparison with nontarget DNA sequences. Qualitative comparisons between ER binding to the VitERE and other sequences in gel shift assays clearly indicate an ability of the ER to distinguish between sequences (Darwish et al., 1991; Klein-Hitpass et al., 1989; Kumar & Chambon, 1988). However, these studies did not determine the relative binding affinity between sequences. Peale et al. (1988) obtained an equilibrium dissociation constant (K_d) of 0.5 nM for ER binding the VitERE and a K_d of 0.2 μM for the plasmid containing the VitERE. Quantitative binding studies from this laboratory (Murdoch et al., 1990) employed the VitERE and the 2 bp mutation of this sequence that failed to mediate an estrogen response as described by Klock et al. (1987, MutERE). The difference in ER affinity for these two sequences was 250-fold in that study. The studies to date indicate an ability of ER to discriminate between sequences, but quantitatively do not show the required degree of discrimination predicted. There has been considerable controversy in the literature regarding the role of hormone in steroid receptor-DNA interactions, but recent work has shown that both the occupied and unoccupied ER can bind DNA with the same high affinity and specificity in vitro [Bagchi et al., 1990; see Murdoch et al. (1990) and references cited therein].

Protein-DNA binding interactions are highly dependent upon salt concentrations, with the general observation that binding affinities decrease with increasing salt concentration [see Record et al. (1976) and references cited therein]. The key question to be addressed here is not the absolute affinity

† This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin—Madison, by National Institutes of Health Grants HD08192 and HD07259, and by the National Foundation for Cancer Research (awarded to J.G.). F.E.M. was the recipient of *NIH* Fellowship HD07136.

* Address correspondence to this author at the Department of Biochemistry, University of Wisconsin—Madison, 420 Henry Mall, Madison, WI 53706.

[1] Abbreviations: ER, estrogen receptor(s); ERE, estrogen response element(s); VitERE, ERE derived from the *Xenopus* vitellogenin A2 gene; MutERE, 2 bp mutation of VitERE; KGlu, potassium glutamate; Tris-HCl, tris(hydroxymethyl)aminomethane titrated with hydrochloric acid to the indicated pH; DTT, dithiothreitol; ^{3}H-E$_2$, 17β-[2,4,6,7-^{3}H]-estradiol; bp, base pair(s); HPLC, high-performance liquid chromatography; ABCD assay, avidin-biotin complexed with DNA assay; RBA, relative binding affinity.

0006-2960/91/0430-10838$02.50/0