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11. The semi-intact preparation (n = 11) was similar, but the caudal two-thirds of the body was left freely moving behind the dissected brainstem and rostral spinal cord. Teflon-coated stainless steel microwires (50 μm in diameter) were inserted into the muscle for EMG recording.
12. Trigeminal inputs to RS neurons in lampreys are mediated by excitatory and inhibitory amino acids (*6*, *20*).
13. Consistent with this observation, we recently recorded NMDA-induced plateau potentials, which are resistant to tetrodotoxin, in RS neurons.
14. Reticulospinal neurons were retrogradely labeled in vitro for 24 to 48 hours by placement of Calcium Green–Dextran (10,000 MW; Molecular Probes) on the rostral end of the sectioned spinal cord at the 1–2 segment level. Labeled cells were imaged on a Nikon epifluorescent microscope and recorded with an intensified charge-coupled device camera at a rate of one to two images per second. Calcium responses are expressed as relative changes in fluorescence ($\Delta F/F$ %). M. J. O'Donovan, S. Ho, G. Sholomenko, W. Yee, *J. Neurosci. Methods* **46**, 91 (1993); A. D. McClellan, D. McPherson, M. J. O'Donovan, *Brain Res.* **663**, 61 (1994); D. A. Nelson and L. C. Katz, *Neuron* **15**, 23 (1995); D. M. O'Malley, Y.-H. Kao, J. R. Fetcho, *Neuron* **17**, 1145 (1996).
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Structural Plasticity in a Remodeled Protein-Protein Interface

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Remodeling of the interface between human growth hormone (hGH) and the extracellular domain of its receptor was studied by deleting a critical tryptophan residue (at position 104) in the receptor, creating a large cavity, and selecting a pentamutant of hGH by phage display that fills the cavity and largely restores binding affinity. A 2.1 Å resolution x-ray structure of the mutant complex showed that the receptor cavity was filled by selected hydrophobic mutations of hGH. Large structural rearrangements occurred in the interface at sites that were distant from the mutations. Such plasticity may be a means for protein-protein interfaces to adapt to mutations as they coevolve.

Protein-protein interfaces are usually large and elaborate, consisting of 10 to 40 contact side chains, each of which interdigitates with several others across the interface (*1*). The contact side chains are often presented from discontinuous segments of each polypeptide chain. Given the complexities of these interactions, we wondered how a functionally disruptive mutation on one side of the interface could be complemented by mutations in its binding partner. This is a challenge that nature faces as protein complexes coevolve.

We studied this problem by phage display using the high-affinity interface between hGH and the extracellular domain of its receptor (hGHbp), members of a cytokine-receptor superfamily (*2*). The hormone and receptor initially form a tight 1:1 complex (dissociation constant K_d = 0.3 nM), and the x-ray structure of this complex is known to high resolution (*3*). There are about 30 contact side chains on each side of this interface, but alanine-scanning mutagenesis has shown that only a small set of primarily hydrophobic contacts at the center of the interface dominate affinity (*4*, *5*). This energetic "hot spot" on the recep-

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tor includes a tryptophan residue at position 104 (W104) (6) that inserts into a groove in hGH (Fig. 1). When W104 is mutated to alanine (W104A), the binding affinity decreases by a factor of >2500 (5). We isolated a variant of hGH that could bind to the mutant W104A-hGHbp and determined the x-ray structure of the complex to see how the interface adapted.

To isolate a mutant of hGH that could bind to W104A-hGHbp, we randomly mutated hGH at five residues (K168, D171, K172, T175, and F176) that pack against W104 (Fig. 1A). A pool of hGH mutants, containing $\sim 10^7$ independent sequences, was displayed on filamentous phagemid particles (7) and selected for binding to immobilized W104A-hGHbp (8). After seven rounds of binding selection, clones were sequenced and screened for binding to W104A-hGHbp. The most frequently isolated clone, called A1-hGH, was the only one found to bind detectably to W104A-hGHbp in a screening assay (9). This variant contained five mutations (K168R, D171T, K172Y, E174A, and F176Y). One of these, E174A, was not encoded by the mutagenic oligonucleotide. Such spurious mutations usually have a strong selective advantage (10). Three of the selected residues (D171T, E174A, and F176Y) were similar to previously selected variants of hGH (D171S, E174S, and F176Y) that bound more tightly to wild-type hGHbp (11).

The affinity for binding of the A1-hGH variant to W104A-hGHbp (12) was dramatically improved by the five mutations (K_d = 14 nM), as compared with wild-type hGH binding to W104A-hGHbp (K_d >1000 nM). To identify the mutations in A1-hGH that most affected binding, we reverted each to their wild-type counterpart and measured their binding affinities to W104A-hGHbp (12). The arginine selected at position 168 and the tyrosine selected at position 176 made only minor contributions to affinity: their reversion back to lysine and phenylalanine, respectively, only reduced affinity up to twofold from the A1 varint (K_d = 33 nM and 21 nM, respectively). However, each of the other three selected mutations (D171T, K172Y, and E174A) caused 15 to >300-fold reductions in affinity when reverted to their wild-type residue (K_d > 5000 nM, 200 nM, and 400 nM, respectively). A1-hGH bound much more weakly to the wild-type hGHbp (K_d > 1 μM), showing it to be highly specific for the W104A-hGHbp mutant (9).

To understand the structural basis for these functional effects, we determined the x-ray structure of the complex between A1-hGH and W104A-hGHbp (13). The crystals were isomorphous with the wild-type 1:1 complex (3) and diffracted to 2.1 Å (Table 1), the highest resolution yet obtained for any hGH:hGHbp complex. The structure shows that the 150 Å^3 hole in W104A-hGHbp is filled by the phenolic group of the critical K172Y mutation of A1-hGH (Fig. 1B); in addition, the I103 side chain in W104-hGHbp rotates around to place its isobutyl side chain in the cavity. This rotation allows a shift of the D126 side chain of W104A-hGHbp, which breaks a hydrogen bond to the main chain at position 104 to form a salt bridge with R43 (Fig. 1B). The movement of D126 is probably coupled to the critical D171T change in A1-hGH, because retention of aspartate at position 171 would cause electrostatic repulsion at D126 of W104A-hGHbp (Fig. 1). Indeed, the binding affinity of a T171A mutation is reduced twofold compared with A1-hGH (K_d = 27 nM), consistent with the preference for a neutral residue over Asp at position 171 for binding to W104A-hGHbp.

The E174A mutation in hGH induces another set of concerted changes (Fig. 1B). In the wild-type complex, E174 makes two hydrogen bonds: one to H18 of hGH and the other to N218 of hGHbp. When E174 is mutated to Ala, H18 rotates to avoid clashing with N218, which allows the hormone and receptor to move closer together (Fig. 1B). The other two selected residues (K168R and F176Y) do not make new contacts with hGHbp, consistent with their

Table 1. Crystallographic statistics for the complex between A1-hGH and W104A-hGHbp. The 2.1 Å data were collected from flash-frozen crystals at Cornell High Energy Synchrotron Source (CHESS) with an Area Detector Systems Corporation charge-coupled device detector positioned at 175 cm with the direct beam in the corner, and data were reduced with DENZO and SCALEPACK (20). Solution of the structure was done by molecular replacement with coordinates from the 2.6 Å resolution 1:1 complex structure (3). The initial refinement was done in X-PLOR (21) and completed by using maximum likelihood in the Refmac package of the CCP4 suite (22). No σ cutoff was used. The final model includes the ordered residues for A1-hGH (residues 3 to 48, 51 to 129, 136 to 148, and 155 to 191) in complex with W104A-hGHbp (residues 32 to 52, 61 to 72, 79 to 236).

Data	Value
Data collection	
Source	CHESS, beam line F1
Space group	$P4_32_12$
Unit cell (Å)	$a = b$ = 66.5, c = 231.7
Resolution (Å)	100 to 2.1
R_{merge} (%)*	5.7
Completeness (%)	91.8
Total reflections	133,933
Unique reflections	28,276
Redundancy	4.7
Anisotropic correction (Å^2)	12
Model refinement	
Resolution (Å)	7 to 2.1
Unique reflections	24,290
rmsd in bonds (Å)†	0.014
rmsd in angles (deg.)†	2.0
Average *B*-factor for protein atoms (Å^2)	27.7
rmsd in *B*-factor of bonded atoms (Å^2)†	2.6
R (%)‡	19.0
R_{free} (%)§	26.2
Waters	382

*$R_{merge} = \Sigma_{hkl}|I - \langle I\rangle|/\Sigma_{hkl} I$, where I is the intensity of unique reflection hkl, and $\langle I\rangle$ is the average over symmetry-related observations of unique reflection hkl. †The root-mean-square deviation (rmsd) in bond lengths and angles is the rmsd from the ideal stereochemical values. The rmsd in the *B*-factor is the rmsd in the temperature factors between bonded atoms. ‡$R = \Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. §R_{free} is R using a set of reflections sequestered before refinement.

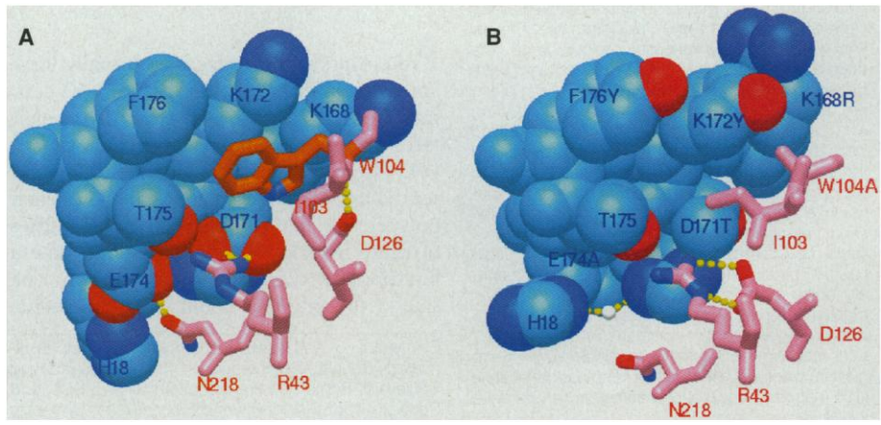

Fig. 1. Residues in hGHbp (rendered in pink or orange sticks) that contact hGH (in blue space-filling rendition) in the 1:1 wild-type complex (**A**) or the complex between A1-hGH and W104A-hGHbp (**B**). Local groups where hydrogen bonds are different between the complexes are shown as yellow dashed lines.

having little effect on binding when reverted back to their wild-type residues.

Overall, the combination of mutations with conformational changes nearly fill the 150 Å^3 cavity introduced by the W104A mutation. A hole of about 20 Å^3 is left between Y172 and Y176 of the hormone and I103 of hGHbp; a hole of about 40 Å^3 is created by the movement of the K168R side chain of hGH. These small cavities, which are not seen filled with ordered solvent, may be disruptive to affinity by analogy to destabilizing mutations in T4 lysozyme (*14*); these cavities may account for the reduced affinity of the complex of A1-hGH with W104A-hGHbp relative to the wild-type (K_d = 14 nM compared with 0.3 nM, respectively).

Remarkably, the local structural changes near the site of the W104A mutation induce substantial global changes that affect the entire interface. The five mutations allow the hormone to move closer to hGHbp in the vicinity of the W104A mutation, with the Cα of K168 1.1 Å closer to the Cα of W104A. This movement is accompanied by a 5° rotation of A1-hGH relative to the receptor. A1-hGH slides across the receptor, pivoting at a point near G168 of hGHbp. The global changes that radiate outward from the pivot point necessitate substantial rearrangement in both main-chain and side-chain contacts throughout the interface. Some of the main-chain and side-chain van der Waals and hydrogen-bonding groups that are 15 Å from the epicenter of these mutations move by up to 3 Å relative to their neighbors in wild-type hGHbp (Fig. 2A). As a result, four hydrogen bonds are lost and three new ones are gained between unchanged atoms in the mutant complex (Fig. 3). For example, the 3 Å salt bridge between K41 and E127 in the wild-type complex, which is 11 Å from the α-carbon of W104, has moved apart by 2.4 Å in the mutant complex (Fig. 3A). This gap is filled by two ordered waters, one of which mediates interactions between this charged pair. The salt bridge in the wild-type complex between R64 and D164, 17 Å from W104, is replaced by one between R64 and E44 in the mutant complex with commensurate changes in ordered waters (Fig. 3B).

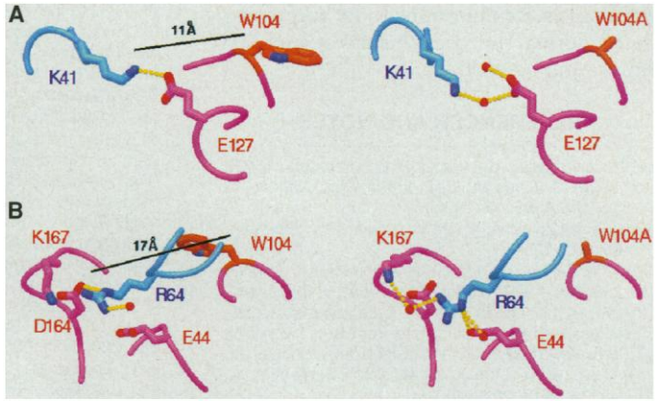

Fig. 3. Two views (**A** and **B**) of nonmutated contact residues that change salt bridge partners in the A1-hGH and W104A-hGHbp complex (right) relative to the wild-type complex (left). Hormone residues are in blue, and receptor residues are in pink, except for position 104 which is in orange.

In contrast, large domain movements and detailed changes in the interface were not seen in another mutant complex in which three functionally inert residues in hGH (F25, Y42, and Q46) were simultaneously mutated to alanine (*15*). These mutations, which are near the periphery of the interface, induced only small movements near the sites of mutation (Fig. 2B); two hydrogen bonds were lost and none were gained.

The structures and binding regions for a number of hematopoietic cytokines and their receptors are now known (*2*, *16*). One of the notable features is that although the same general regions of the hormone and receptor are involved in binding, the exact nature of the contacts is very different. For example, W104 of hGHbp is conserved in only a few of the cytokine receptors, particularly the prolactin receptor, which shares 30% sequence identity with hGHbp and also binds hGH. Interestingly, the structure of the complex between hGH and the prolactin receptor also shows substantial differences in domain orientation compared with hGHbp (*16*).

These studies reveal how large, functional changes can be rescued by a limited number of mutations. The mutations were accommodated by remarkably large movements in unaltered side-chain and main-chain contacts throughout the interface. Structural changes induced by mutations have been seen in x-ray structures of single-domain proteins; for example, when core residues were mutated (*17*) or when surface residues were inserted or deleted in α helices of T4 lysozyme (*18*) or staphylococcal nuclease (*19*). Here, it is striking that such large changes are observed over a large interface in a bimolecular protein complex, requiring coordinated changes in both components, and that they involve very specific interactions such as hydrogen bonds and salt bridges. These results suggest that structural plasticity is nascent in protein-protein interfaces and provides a

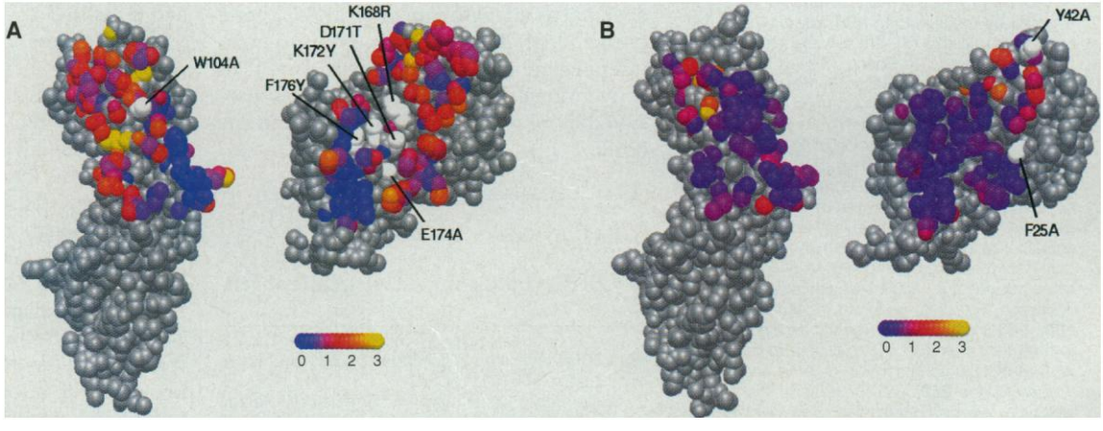

Fig. 2. Space-filling models showing movements in contact residues for A1-hGH in complex with W104A-hGHbp (**A**) and F25A, Y42A, Q46A-hGH in complex with hGHbp (**B**) compared with the wild-type complex. hGHbp is shown on the left and the hormone on the right. All contact residues are colored such that those that were mutated are in white, and those that moved relative to the wild-type complex by 0, 1, 2, and 3 Å are shown in a sliding scale from purple to red to orange to yellow, respectively. Changes were calculated by taking each atom that is in the interface of the wild-type or mutant complex and calculating the average change in distance to all atoms across the interface that are within 5 Å.

mechanism for mutations to be accommodated during the coevolution of high-affinity binding partners.

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6. The single letter abbreviations for amino acid residues are as follows: 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. Mutants are identified by the wild-type residue, followed by its position, and the mutant residue. Multiple mutants are indicated by a series of single mutants separated by commas.
7. The library was constructed by site-directed mutagenesis [T. A. Kunkel, K. Bebenek, J. McClary, *Methods Enzymol.* **204**, 125 (1991)] by substituting NNS (N = A, G, C, or T; S = G or C) for the codon to be randomized. The library that randomized hGH at codons 168, 171, 172, 175, and 176 was made by mutagenizing the plasmid pH0753 with the oligonucleotide 5′-CTG CTC TAC TGC TTC AGG NNS GAC ATG NNS NNS GTC GAG NNS NNS CTT CGA ATC GTG CAG TGC CGC TCT-3′. The initial library size was 2×10^7. The plasmid pH0753 was derived from phGHam-g3 [H. B. Lowman, S. H. Bass, N. Simpson, J. A. Wells, *Biochemistry* **30**, 10832 (1991)] by mutation of the base at position 419 from C to G in order to eliminate an Xba I restriction site.
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13. We purified A1-hGH and W104A-hGHbp from an *E. coli* fermentation culture using ion exchange and hydrophobic interaction columns. The two proteins were combined and purified as a complex on a gel filtration column. Crystals were induced by streak seeding with crystals of the F25A, Y42A, Q46A-hGH:hGHbp complex (*15*) (~5 mg/ml) in 50 mM bis-tris (pH 6.5) and 17 to 20% saturated ammonium sulfate. Crystals were frozen in liquid nitrogen for data collection.
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A Low-Barrier Hydrogen Bond in the Catalytic Triad of Serine Proteases? Theory Versus Experiment

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Cleland and Kreevoy recently advanced the idea that a special type of hydrogen bond (H-bond), termed a low-barrier hydrogen bond (LBHB), may account for the "missing" transition state stabilization underlying the catalytic power of many enzymes, and Frey *et al.* have proposed that the H-bond between aspartic acid 102 and histidine 57 in the catalytic triad of serine proteases is an example of a catalytically important LBHB. Experimental facts are here considered regarding the aspartic acid–histidine and *cis*-urocanic H-bonds that are inconsistent with fundamental tenets of the LBHB hypothesis. The inconsistencies between theory and experiment in these paradigm systems cast doubt on the existence of LBHBs, as currently defined, within enzyme active sites.

The H-bond inherently involves the sharing of hydrogen atoms to varying extents with other atoms (*1*). This sharing is often depicted as a chemical equilibration or resonance hybridization of structures such as **1** and **2** (Eq. 1). Proton sharing can also be depicted as a lengthening of the A-H bond of the donor, **1**, as if the proton were in an intermediate stage of transfer to B (*2*). In conventional H-bonds the H atom is associated more with one heteroatom than the other.

$$\underset{\mathbf{1}}{A\text{–}H\cdots:B} \rightleftharpoons \underset{\mathbf{2}}{A:\cdots H\text{–}B} \qquad \underset{\mathbf{3}}{A\cdots H\cdots B} \quad (1)$$

LBHBs are distinguished from conventional H-bonds by equal proton sharing between the heteroatoms. LBHBs can be double-welled, depicted as **1** and **2** contributing equally to the system; or single-welled, depicted as a single structure with the proton residing at a point equidistant between A and B, as in **3** (Eq. 1).

LBHBs were first observed in the gas phase (*1*, *3*), where evidence for their existence and strength is persuasive. Schowen first proposed that LBHBs may exist within the protected interior of proteins (*4*). Promotion of this idea by Cleland and Kreevoy (*5*) and Frey *et al.* (*6*) has led to its substantial (*7*), though not universal (*8*), acceptance. Physicochemical parameters used to identify LBHBs include (i) extreme low-field ^{1}H nuclear magnetic resonance (NMR) chemical shifts [$\delta > 16$ parts per million (ppm)]; (ii) deuterium isotope effects on low-field ^{1}H resonances; (iii) low (<1.0) isotopic fractionation factors; and (iv) deuterium isotope effects on infrared and Raman frequencies (*1*)—the most unambiguous of

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