observation that the mitogenic potency of RET varies in different target cells (*14*).

These data establish that mutations in MEN2A and MEN2B convert *RET* into a dominant transforming gene. They also show the underlying mechanism to be a dominant oncogenic conversion rather than a loss of suppressor function. This finding is based on the observations that MEN2A and MEN2B mutations concomitantly activate the intrinsic kinase and transforming ability of RET and that mutant RET is enzymatically activated in the presence of a coexpressed normal allele as was shown in TT cells. Thus, the oncogenic conversion of *RET* illustrates that a dominant transforming gene can have a causal role in human hereditary neoplasia. In addition, our identification of the molecular mechanisms of RET activation provides the basis for therapeutic strategies in MEN2A, MEN2B, and FMTC.

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5. We assembled the open reading frame (ORF) of proto-*RET* by starting from clones derived from an SK-N-BE complementary DNA (cDNA) library (corresponding to the extracellular portion of RET) and a fragment of a described EGFR-*RET* construct (*14*), corresponding to the intracellular portion of RET. We completely sequenced the resulting cDNA, which encodes the short isoform of the RET protein (*3*), on both strands and found it to match exactly the published sequence of wild-type *RET* (*3*). The proto-*RET* ORF was then cloned in the LTR-2 plasmid (*18*) to yield the LTR-*ret* eukaryotic expression vector. Using the RT-PCR, we cloned cDNA fragments with the desired mutations from RNA obtained from neoplastic tissues of individuals with MEN2A and MEN2B. Nucleotide sequence analysis confirmed that no other mutations were present in the cDNA fragments. For the MEN2A expression vectors, Nde I–Eco RI fragments [corresponding to positions 1717 to 2348 of the proto-*RET* sequence (*3*)] from the mutated cDNAs were individually substituted for the analogous fragment in LTR-*ret*. For the MEN2B expression vector, a Bgl II–Bcl I fragment (positions 2765 to 2972 of proto-*RET*) was substituted for the analogous fragment in LTR-*ret*. A second cycle of sequence analysis confirmed the introduction of the expected mutations. Because all of the mutations were introduced directly into the LTR-*ret* vector, all constructs were identical except for the specified mutations, thus allowing comparison of their transforming abilities.
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9. The immunocomplex kinase assay was performed as in (*16*). Wild-type and mutant RETs were immunoprecipitated with the antibody to RET (*14*). The final kinase mixture contained 0.1% Triton X-100, 20 mM Hepes (pH 7.5), 150 mM NaCl, 15 mM $MgCl_2$, 15 mM $MnCl_2$, and 20 μCi of γ-^{32}P–labeled adenosine triphosphate (3000 Ci/mmol). In the experiment shown, the reaction was for 20 min at room temperature. Similar results were obtained under initial conditions (1 min at 4°C).
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A Hot Spot of Binding Energy in a Hormone-Receptor Interface

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The x-ray crystal structure of the complex between human growth hormone (hGH) and the extracellular domain of its first bound receptor (hGHbp) shows that about 30 side chains from each protein make contact. Individual replacement of contact residues in the hGHbp with alanine showed that a central hydrophobic region, dominated by two tryptophan residues, accounts for more than three-quarters of the binding free energy. This "functional epitope" is surrounded by less important contact residues that are generally hydrophilic and partially hydrated, so that the interface resembles a cross section through a globular protein. The functionally important residues on the hGHbp directly contact those on hGH. Thus, only a small and complementary set of contact residues maintains binding affinity, a property that may be general to protein-protein interfaces.

Specific protein-protein interactions are critical events in most biological processes. A number of crystallographic studies have shown that the binding interfaces between proteins are generally large (600 to 1300 Å^2) and include many intermolecular contacts, involving 10 to 30 side chains from each protein (*1–3*). However, structural analysis alone cannot show whether all of these contacts are important for tight binding. A complete understanding of the chemistry of protein-protein association also requires a functional map of each binding surface, to reveal to what extent each contact contributes to the overall free energy of binding.

The initial event in signaling through the hGH receptor is the binding of the extracellular domain (hGHbp) to site 1 of hGH to form a high-affinity 1:1 complex (dissociation constant, K_d, of 0.3 nM, corresponding to a binding free energy, ΔG, of −12.3 kcal/mol) (*4, 5*). The crystal structure of this complex (*3, 6*) shows that on each protein a surface area of about 1300 Å^2 becomes buried (defined by calculated inaccessibility to solvent) (*7*), including 33 side chains on the receptor (Fig. 1A). We mutated each of these side chains individually to alanine, except for G168 and the C108–C122 disulfide (*8*). Substitution by alanine deletes all interactions made by atoms beyond the β carbon and should reveal the contribution to binding energy made by the removed portion of the side chain (*9*). Each mutant hGHbp was expressed in *Escherichia coli*, purified to >90% homogeneity, and assayed for hGH binding affinity (*10, 11*).

Fewer than half of the mutations caused substantial loss in binding affinity (Fig. 1B). By far the greatest reductions in affinity (>4.5 kcal/mol) occurred on substituting two tryptophan residues, W104 and W169 (*12*). Large effects (1.5 to 3.5 kcal/mol) were also seen for alanine substitutions at other hydrophobic residues (I103, I105, P106, and I165), and generally smaller effects (1 to 2 kcal/mol) were seen for some charged residues (R43, E44, D126, E127, and D164). This subset of 11 contact residues, which we term the functional epitope, maps to a con-

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tiguous patch at the center of the contact region (Fig. 2A) in which the hydrophobic residues form a core flanked by the charged groups (Fig. 2B). Several of the hydrophobic residues (for instance I103) are in fact largely buried beneath the W104 and W169 side chains, suggesting that their main functional contribution is indirect (*13*). The functional epitope is surrounded by the residues whose mutation to alanine has little effect on binding affinity and in some cases (V171A) even increases it. This peripheral nonfunctional region corresponds to 46% of the area buried upon binding.

A similar strategy has previously been used to determine the functional profile on the hormone side of the interface (*14*). Eight out of 31 side chains in hGH accounted for approximately 85% of binding energy, and again, over half of the residues made no substantial contribution to binding affinity. Now that both sides of the interface have been mutated systematically, the two functional epitopes can be compared (Fig. 3A). This reveals a striking complementarity—the energetically critical and unimportant regions on one molecule match those on the other. Most of the important residues on hGH are involved in forming a hydrophobic pocket that closely docks the side chains of W104 and W169 from the hGHbp (Fig. 3B). Although the electrostatic interactions are less important for affinity, direct intermolecular contacts are made between the most functionally important charged groups (Fig. 3C). Thus, the two proteins interact through complementary functional epitopes that pack together to form a tightly packed hydrophobic core, surrounded by five intermolecular salt bridges and hydrogen bonds. In this respect, the energetics of the binding interface are reminiscent of a cross section through a folded protein—hydrophobic residues are inside and hydrophilic residues are outside (*15*).

We wished to understand better why some contacts are more important than others. Structural parameters such as buried surface area (*16*) did not correlate well with the energetic importance of individual residues at this interface (Fig. 1), nor did the number of van der Waals contacts made by each side chain (*17, 18*), their crystallographic temperature factors (*19*), or solvation parameters calculated with a semi-empirical method (*20*). We presume that for the nonfunctional residues, the energetic cost of desolvation and side chain rearrangement offsets, and in some cases exceeds, the energy gained through the intermolecular interactions. The structure of the complex does not allow issues of solvent and side chain structure in the free components to be addressed, and neither protein has been crystallized alone. Nonetheless, the complexed structure does show that the intermolecular packing at the functionally critical regions is frequently better than that at the functionally unimportant ones: in particular, there are cavities between the van der Waals surfaces of many of the polar residues. These cavities are filled with well-ordered water molecules that par-

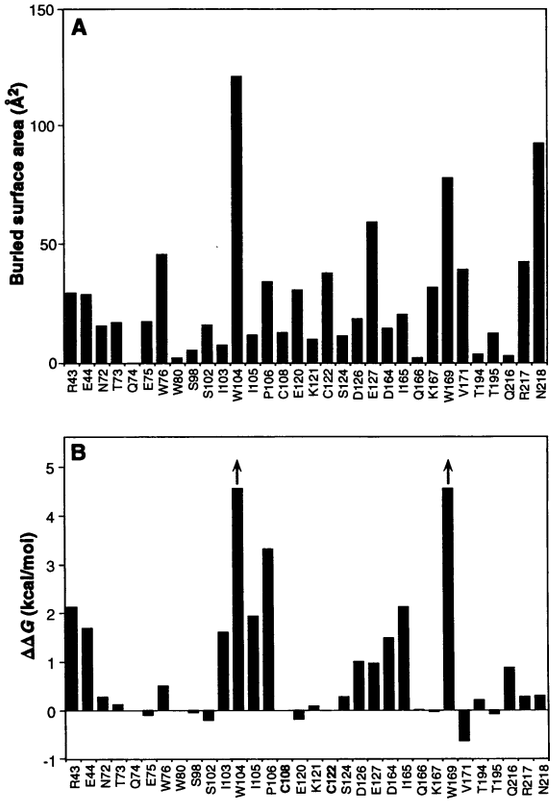

Fig. 1. Contribution of only a subset of contact residues to net binding energy. (**A**) Loss of solvent-accessible area (*7*) of the side chain portion of each residue in the hGHbp on forming a complex with hGH. (**B**) Difference in binding free energy between alanine-substituted and wild-type hGHbp $(\Delta\Delta G)_{mut\text{-}wt}$ at contact residues (*5*). Negative values indicate that affinity increased when the side chain was substituted by alanine.

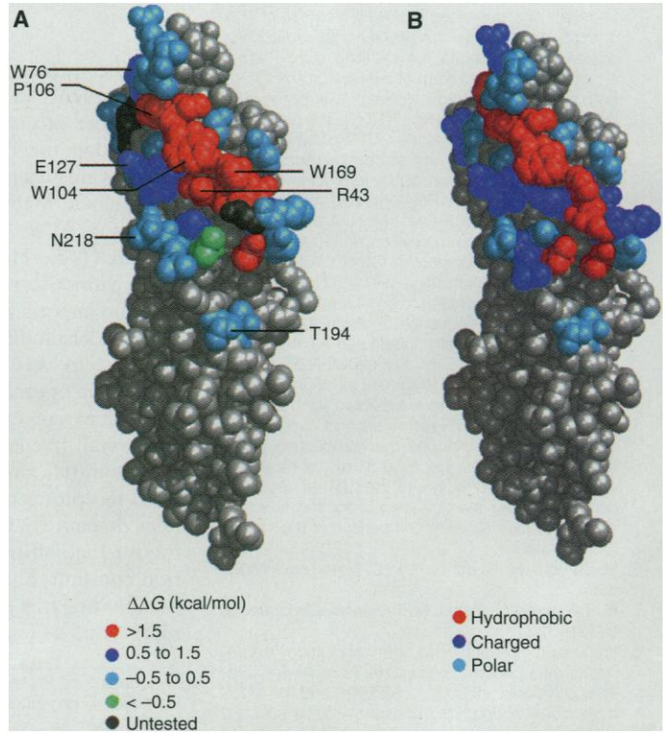

Fig. 2. Mapping of structural and functional epitopes for binding of hGH site 1 onto the structure of hGHbp, derived from the 1:1 complex (*6*). (**A**) The functional epitope on the hGHbp. Residues are color-coded according to the loss of binding free energy upon alanine substitution: red, >1.5 kcal/mol; blue, 0.5 to 1.5 kcal/mol cyan, −0.5 to +0.5 kcal/mol; green, <0.5 kcal/mol (a substantial increase in affinity upon substitution); and gray, untested. (**B**) The structural epitope. All side chains on the hGHbp that lose accessibility to solvent when hGH binds through site 1 (*7*) are colored according to their physicochemical properties: red, hydrophobic; blue, charged; and cyan, polar. The structural epitope forms a near-continuous patch, yet is highly discontinuous, comprising side chains from seven separate parts of the polypeptide chain. These and other similar figures were drawn with the conic subroutine of MIDAS-plus (*33*).

ticipate in intermolecular hydrogen-bonding networks involving both main chain and side chain atoms (Fig. 4).

Ordered water molecules have been observed in other protein-protein interfaces (*1*, *21*, *22*), and in one case the presence of water in equivalent positions in the bound and unbound states was demonstrated (*21*). Some other complexes of proteins with small molecules (*23*) or with DNA (*24*) appear to have trapped waters as well. The hGH-hGHbp complex structure we used (*6*) was at moderate resolution (2.6 Å), so that only the most ordered waters could be accurately placed. Nevertheless, the correlation of solvation with the functional neutrality of side chains suggests that the interactions of these trapped waters (at least with side chains) do not contribute much to net binding energy. Rather, the water molecules appear to fill gaps between imperfectly packed regions of the interface and form hydrogen bond interactions that are approximately isoenergetic with those for the two unbound proteins. The generality of this correlation must await the systematic functional analysis of other structurally well-characterized interfaces.

The binding free energy between hGH and hGHbp could be generated through only a few strong interactions, or from the accumulation of many weaker contacts over the entire interface. Our data indicate that the hGH-hGHbp interaction occurs in the former manner. Functional studies on other protein-protein complexes also indicate that a small number of residues can confer tight binding affinity. For example, mutational analysis of antibodies (*18*, *25*) or protein antigens (*26*) has shown that only 3 to 10 side chains can account for most of the binding energy, although the structures of complexes between antibodies and protein antigens (*2*) show that typically between 14 and 21 residues are in contact. These conclusions support theoretical predictions that only a few interactions may be important for tight binding (*27*). For the hGH-hGHbp interaction, we found that these are predominantly interactions between hydrophobic interface regions. Binding sites in other protein-protein complexes of known structure often include patches of high surface hydrophobicity (*28*); perhaps these are also sites of energetic importance.

Although the peripheral contact residues in the hGH-hGHbp complex do not contribute much to net binding energy, they probably have other important roles. Peripheral electrostatic interactions increase the rate of association (*14*) and may be critical for the solubility of the hormone and receptor when uncomplexed. The peripheries of interfaces can also contribute substantially to the specificity of binding (*29*), by repulsion of nontarget molecules through unfavorable electrostatic or steric interactions, or both. They may also be required for other binding activities. For example, hGH interacts with the human prolactin receptor using the same contact residues as for the hGH receptor (*30*); but different, although overlapping, subsets of residues are functionally critical for binding (*29*). Thus, a single set of contact residues can bind two different targets, using smaller and alternative functional epitopes.

The structures of many oligomeric proteins (*31*) and protein-protein complexes (*1–3*, *6*, *21*, *22*, *30*, *32*) show them to have generally large and flat interfaces, making the rational design of small molecule mimics of these surfaces a daunting prospect. However, if proteins generally interact through much smaller functional epitopes, as seen here, then the task of designing small inhibitors might be greatly simplified:

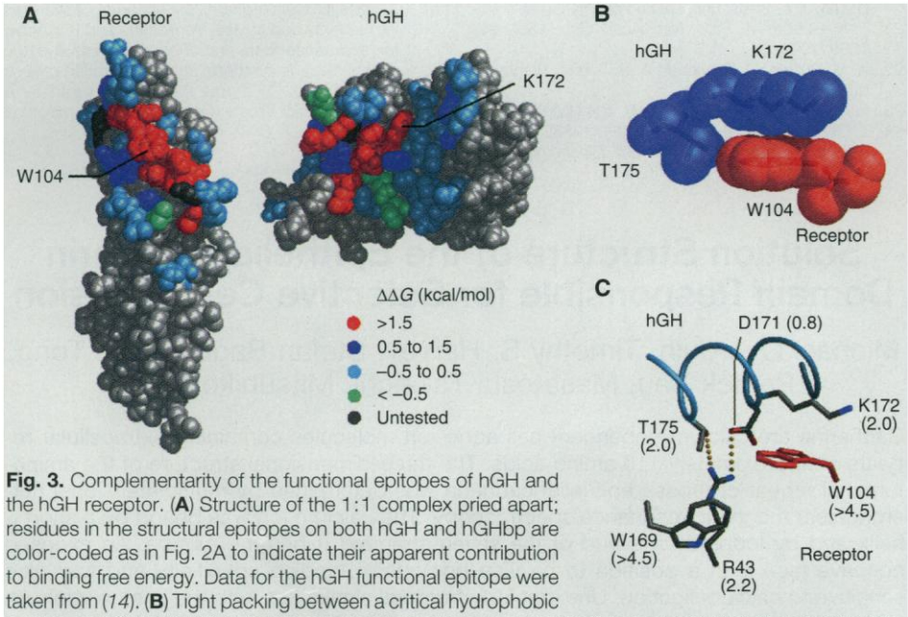

Fig. 3. Complementarity of the functional epitopes of hGH and the hGH receptor. (**A**) Structure of the 1:1 complex pried apart; residues in the structural epitopes on both hGH and hGHbp are color-coded as in Fig. 2A to indicate their apparent contribution to binding free energy. Data for the hGH functional epitope were taken from (*14*). (**B**) Tight packing between a critical hydrophobic residue on hGHbp (W104) and the alkyl portions of two important residues on hGH (K172 and T175). The van der Waals surfaces are shown. In this view, the side chain of hGH residue D171 would pack behind the indole ring of W104, and that of hGH F176 would make contacts at the front to complete the binding pocket for W104; for clarity these residues have been omitted from the figure. (**C**) Broader view of the same area shown in (B), showing direct electrostatic contacts between the most important charged contact residue on hGHbp (R43) and two charged or polar residues on helix 4 of hGH (D171 and T175). The alkyl portion of the R43 side chain packs beneath the indole ring of W169 on the hGHbp; thus, R43 may play an indirect structural role by supporting W169, in addition to interacting directly with D171 and T175. The observed energetic contributions of each side chain are indicated in parentheses (in units of kcal/mol). The apparent contribution of hGH F176 (not shown; see above) is 1.9 kcal/mol. Data for hGH were taken from (*14*).

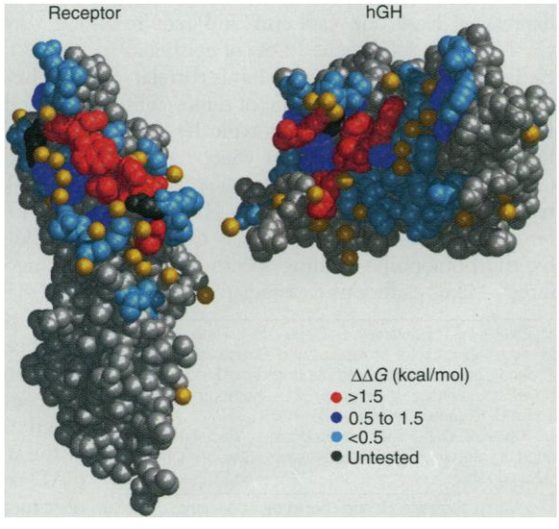

Fig. 4. Presence of ordered water molecules in interface regions that are unimportant for binding affinity. The separated complex is shown with contact residues color-coded according to their functional importance as in Fig. 2A except that, for clarity, all the less important residues (contributing less than +0.5 kcal/mol), including those for which substitution increases affinity, are colored cyan. Ordered water molecules within 4 Å of each protein are shown in yellow (thus, most of the water molecules bound to the hGHbp on the left are also shown bound to hGH on the right).

a mimic of the smaller functional epitope may suffice. Our findings suggest starting points for these design strategies.

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6. Our analysis is based on a crystal structure of the 1:1 complex at 2.6 Å resolution [A. M. de Vos and M. Ultsch, in preparation]. Compared with the 2:1 structure at 2.8 Å resolution that included a second receptor (*3*), the 1:1 structure shows the previously disordered loop region T73 to E75 in the hGHbp and many ordered water molecules. In other respects the interface is essentially unchanged, except that the flexible COOH-terminus of hGH makes different contacts (with receptor residues T194 and T195).
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11. Most mutants were engineered, expressed in *E. coli*, and purified by hGH affinity chromatography essentially as described [(*10*); G. Fuh *et al.*, *J. Biol. Chem.* **265**, 3111 (1990)]. Some mutants had been constructed previously (*10*). Mutants W104A, P106A, and W169A were purified by ion-exchange chromatography and hydrophobic interaction chromatography on a BIOcad workstation (Perseptive Biosystems). Binding affinities for hGH were determined by competitive displacement with ^{125}I-labeled hGH as the tracer [S. A. Spencer *et al.*, *ibid.* **263**, 7862 (1988)] except that monoclonal antibody (mAb) 5 [R. Barnard, P. G. Bundesen, D. B. Rylatt, M. J. Waters, *Endocrinology* **115**, 1805 (1984)] was used to precipitate hGH-hGHbp complexes. This mAb blocks dimerization of hGHbp [B. C. Cunningham *et al.*, *Science* **254**, 821 (1991)] so that simple 1:1 binding affinity can be accurately determined. Any discrepancies between the present data and other measurements of affinities (*10*) can be ascribed to the previous use of mAb 263, which does not block dimerization.
12. The affinities of W104A and W169A were below the sensitivity of the assay ($K_d > 1$ μM), which is fixed by the availability of mAb 5 for precipitation. We estimated the affinity of these mutants for hGH to be 1/2500 that of the wild-type hGHbp protein or even lower. The mutations do not appear to introduce global structural disruptions into hGHbp, as their reactivities with a panel of mAbs were unchanged [(*10*); T. Clackson and J. A. Wells, unpublished data]; in addition, the W104A mutant has a native-like circular dichroism spectrum (*10*) and can bind to a variant of hGH engineered to have higher receptor-binding affinity [H. B. Lowman and J. A. Wells, *J. Mol. Biol.* **234**, 564 (1993); S. Atwell and J. A. Wells, unpublished data].
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Solution Structure of the Epithelial Cadherin Domain Responsible for Selective Cell Adhesion

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Cadherins are calcium-dependent cell adhesion molecules containing extracellular repeats of approximately 110 amino acids. The three-dimensional structure of the amino-terminal repeat of mouse epithelial cadherin was determined by multidimensional heteronuclear magnetic resonance spectroscopy. The calcium ion was bound by a short α helix and by loops at one end of the seven-stranded β-barrel structure. An exposed concave face is in a position to provide homophilic binding specificity and was also sensitive to calcium ligation. Unexpected structural similarities with the immunoglobulin fold suggest an evolutionary relation between calcium-dependent and calcium-independent cell adhesion molecules.

Selective interactions between cells that lead to morphogenesis require the action of cell adhesion molecules (CAMs). The cadherin and immunoglobulin (Ig) CAM superfamilies provide Ca^{2+}-dependent and Ca^{2+}-independent cell adhesion, respectively (*1*). Most vertebrate and some invertebrate cells express at least one cadherin and require Ca^{2+} to form solid tissues. Loss of epithelial cadherin (E-cadherin) expression is correlated with the invasive potential of tumor cells (*2*). The 30 known cadherins typically contain five extracellular repeats (here termed CAD repeats), a single membrane-spanning region, and a cytoplasmic region. The NH_2-terminal CAD (CAD1) repeat is essential for the homophilic binding specificity that directs "like" cadherins to associate (*3*). The cytoplasmic region of E-cadherin (also called uvomorulin) is anchored to cytoskeletal actin microfilaments through catenins (*4*). Here we present the solution structure of the CAD1 domain of E-cadherin spanning amino acids 1 through 104 (referred to hereafter as E-CAD1), determined in the presence of Ca^{2+} from 1793 nuclear magnetic resonance (NMR)–derived structural restraints (*5*).

The structure of E-CAD1 contains seven β strands (βA through βG) and two short α helices (αA and αB) (Fig. 1A). All β strand pairings are antiparallel except for that between βA′ and βG, with a β-barrel topology similar to that of the Ig constant (C) domain (*6*). Bulges in βB and βG contribute to the curling of the β sheet into a barrel shape (Fig. 1B). A proline-proline junction between βA and βA′ bridges the two ends of the sheet. Despite its helical appearance, the backbone dihedral angles in the CD loop are not compatible with a helix. The DE and FG junctions are β hairpin turns. Conservation of the β-barrel fold of E-CAD1 among other CAD repeats is evident from the alignment of structurally critical proline, glycine, and

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