Protein Science (1994), *3*:1697-1705. Cambridge University Press. Printed in the USA.

INVITED PAPER, SPECIAL SECTION IN HONOR OF MAX PERUTZ

Comparison of the intermediate complexes of human growth hormone bound to the human growth hormone and prolactin receptors

ANTHONY A. KOSSIAKOFF, WILLIAM SOMERS, MARK ULTSCH, KERRIE ANDOW, YVES A. MULLER, AND ABRAHAM M. DE VOS

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

(RECEIVED July 25, 1994; ACCEPTED July 28, 1994)

Abstract

The crystal structures of complexes of human growth hormone (hGH) with the growth hormone and prolactin receptors (hGHR and hPRLR, respectively), together with the mutational data available for these systems, suggest that an extraordinary combination of conformational adaptability, together with finely tuned specificity, governs the molecular recognition processes operative in these systems. On the one hand, in the active 1:2 ligand-receptor complexes, 2 copies of the same receptor use the identical set of binding determinants to recognize topographically different surfaces on the hormone. On the other hand, comparing the 1:1 hGH-hGHR and hGH-hPRLR complexes, 2 distinct receptors use this same set of binding determinants to interact with the identical binding site on the ligand, even though few residues among the binding determinants are conserved. The structural evidence demonstrates that this versatility is accomplished by local conformational flexibility of the binding loops, allowing adaptation to different binding environments, together with rigid-body movements of the receptor domains, necessary for the creation of specific interactions with the same binding site.

Keywords: crystal structures; human growth hormone; human prolactin; molecular recognition processes; receptors

Although receptor signaling has been the subject of intense investigation, the mechanistic aspects of ligand-induced receptor activation have only recently been studied and understood at the molecular level (De Vos et al., 1992; Banner et al., 1993). A number of structural classes of cell surface receptors have been characterized. They are generally similar, containing an extracellular portion that binds the activating ligand, a transmembrane polypeptide that may traverse the membrane once (single pass) or several times, and an intracellular or cytoplasmic domain. Binding of the ligand triggers the biological response; the chain of events leading to production of the second message is usually initiated by phosphorylation of the cytoplasmic domain.

Reprint requests to: Abraham M. De Vos, Department of Protein Engineering, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080.

One of the largest receptor families within the category of the single-pass receptors is the hematopoietic superfamily, which has 2 subclasses (Bazan, 1990; Cosman, 1993; Sprang & Bazan, 1993). The receptors in this superfamily direct the growth and differentiation of hematopoietic cells and, as such, are being intensively studied as targets for drug development. As pictured in Figure 1, subclass 2 of the family includes receptors for the cytokine growth factors, growth hormone (GH), prolactin (PRL), several of the interleukins, 2 colony-stimulating factors (granulocyte-macrophage [GM-] and granulocyte [G]-CSF), erythropoietin (EPO), and MPL, the cellular counterpart of the product of the viral *mpl* oncogene product. In the structurally related subclass 1 are receptors for the interferons α and γ, together with tissue factor. In all instances, the mechanism of receptor activation involves receptor clustering initiated by a single copy of the ligand, in some cases bringing together 2 cop-

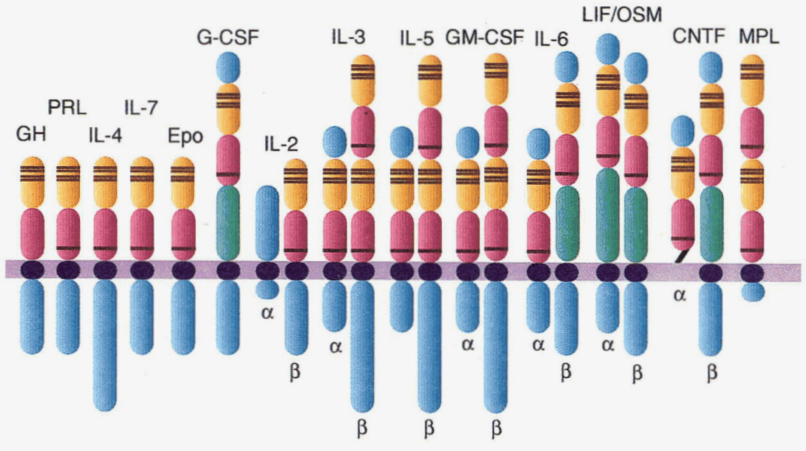

Fig. 1. The hematopoietic receptor superfamily. The extracellular portions are at the top, the cell membrane is represented as a horizontal bar, and the cytoplasmic domains are the blue symbols below the membrane. Individual domains are shown by separate symbols, and extracellular modules with identical colors represent related structural units: pink, fibronectin type III module; yellow, cytokine receptor module; light blue, immunoglobulin module; green, fibronectin type III spacer. The conserved disulfide bonds in the cytokine receptor modules are shown, as is the position of the WSXWS box in the fibronectin type III modules. The receptors are labeled with the name of the ligand: GH, growth hormone; PRL, prolactin; IL-4, interleukin 4; IL-7, interleukin-7; Epo, erythropoietin; G-CSF, granulocyte colony-stimulating factor; IL-2, interleukin 2; IL-3, interleukin 3; IL-5, interleukin 5; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-6, interleukin 6; LIF/OSM, leukemia inhibitory factor/oncostatin M; CNTF, ciliary neurotrophic factor; MPL, the cellular counterpart of the viral *mpl* oncogene product.

ies of the same receptor, in others 2 or 3 distinct receptor chains (Boulay & Paul, 1993; Cosman, 1993; Sprang & Bazan, 1993).

There is no known homology among the cytoplasmic portions of these receptors, although biological signaling involves receptor phosphorylation by JAK-type kinases (Ihle et al., 1994). The classification of the hematopoietic receptors by sequence similarity is consistent with a particular structural motif of their extracellular parts (Bazan, 1990). These extracellular portions include 2 domains containing 7 β-strands each, with a topology similar to that of a fibronectin type III domain. Although some of the receptors contain additional domains, ligand binding is principally driven by the fibronectin-like structures. A common feature of the receptor superfamily is the presence of the sequence Trp-Ser-X-Trp-Ser (where X can be any amino acid), commonly referred to as the WSXWS box. The biological role of this sequence is controversial, but our results suggest that the actual relevant region is significantly larger and presents characteristics much different than those of the pentapeptide alone.

In the cytokine branch of the family, there are also structural similarities among the ligands (De Vos & Kossiakoff, 1992; Kaushandy & Karplus, 1993), which fold in a characteristic 4-helix bundle with 2 sets of parallel helices running in an "up-up-down-down" fashion (Abdel-Meguid et al., 1987). There is no internal sequence similarity in the ligands and no internal symmetry in their 3-dimensional structures. This is noteworthy because the clustering of receptors is effected by a single ligand molecule, even when 2 copies of the same receptor are involved. The crystal structure of human growth hormone (hGH) bound to the extracellular domain of its receptor (hGHR) showed 2 identical receptor molecules, using essentially the same binding determinants, bound to 2 dissimilar sites on the hormone (De Vos et al., 1992). The structure shows that the receptors adapt to these different environments by local conformational changes in some of their binding loops. This case presents an interesting example of molecular recognition quite distinct from the antibody–antigen paradigm, where recognition is developed through sequence differences on the 6 antigen-binding loops.

Biological regulation also requires that the endocrine receptors have a finely honed window of specificity. Whereas PRL does not bind to the GH receptor, hGH binds and activates not only the hGHR, but also the prolactin receptor (hPRLR), resulting in physiological effects otherwise associated with PRL (Chawla et al., 1983). Mutagenesis studies (Cunningham & Wells, 1991) and crystal structures of the hGH–hGHR and hGH–hPRLR complexes (De Vos et al., 1992; Somers et al., 1994) have shown that, in these cases, the ligand uses the same receptor binding site. This raises the question how 2 distinct receptors, sharing only about 28% sequence homology, bind to the same binding site on the ligand.

The extensive mutagenesis analysis performed on the endocrine hormone–receptor systems, coupled with the detailed structural information developed through X-ray studies, offers one of the most complete pictures available of the structure–function relationships of binding and specificity determinants in protein–protein interactions (Somers et al., 1994; for a review, see Wells & De Vos, 1993). What has become clear is that, although the binding interfaces between ligands and receptors are extensive, the majority of the binding energy is developed through interactions among a relatively small number of residues on each molecule. Although in the respective complexes hGH binds to essentially the same residues on the hGHR and hPRLR, the conformations of these receptor residues undergo significant changes to accommodate the ligand (Somers et al., 1994). Furthermore, an important subset of the binding determinants is different between the 2 systems, effectively altering residues providing for each system's specificity.

In this paper, we discuss several aspects of the binding and specificity for the endocrine hormone–receptor systems in the context of the available mutagenesis and structural data. An important element in the formation of the activated complex is the set of interactions between the extracellular portion of the 2 receptors along a section of their C-terminal domains. The relative disposition of the C-terminal domains in the hGH complexes with the hGHR and hPRLR require that significant differences exist between the receptor–receptor interfaces. These differences will be discussed in light of a comparison developed through a model-building experiment. Finally, the structural similarities and differences among the hematopoietic receptors will be discussed by comparing the structures of the hGHR and

hPRLR, which belong to class 1 of the superfamily, to that of tissue factor, a member of class 2.

Hormone–receptor interactions

Significance of the intermediate 1:1 complexes

The structure of the hGH–hGHR complex suggests that the active assembly in the case of the single-chain cytokine receptors is a ternary complex with a stoichiometry of 1 hormone to 2 receptors (Cunningham et al., 1991; De Vos et al., 1992). (We note, however, that the structure of the WSXWS box [see below] suggests that an accessory protein might be involved as well). Mutagenesis analyses have established that receptor activation is a sequential process, the first step always being binding of the receptor to the high-affinity binding site, the so-called site 1 on hGH (Cunningham & Wells, 1989, 1993). The resulting 1:1 complex represents an intermediate step toward formation of the active ternary complex. Only after the 1:1 complex forms can the second receptor bind to the residues in site 2 on hGH together with an interaction with the C-terminal domain of the first receptor (De Vos et al., 1992). The same general scenario appears to hold for the complexes with the PRLR (Fuh et al., 1993).

All mutagenesis and structural studies on the interactions of hGH with its receptors were carried out using the extracellular portions rather than intact receptors. In the case of the hGHR, this portion of the receptor exists naturally as an hGH binding protein in plasma and has an important biological role in the regulation of hGH activity (Baumann et al., 1994). In both the hGH–hGHR and hGH–hPRLR systems, a stable intermediate complex having the stoichiometry 1 hormone to 1 receptor can be readily isolated using the G120R mutant of hGH (Ultsch & De Vos, 1993). However, in contrast to hGH–hGHR, it is hard to isolate or even identify a 1:2 hGH–hPRLR complex in solution. As a result, we were only able to crystallize the 1:1 hGH–hPRLR complex form, and it is this complex whose structure was determined (Somers et al., 1994) and which is the basis for discussion here.

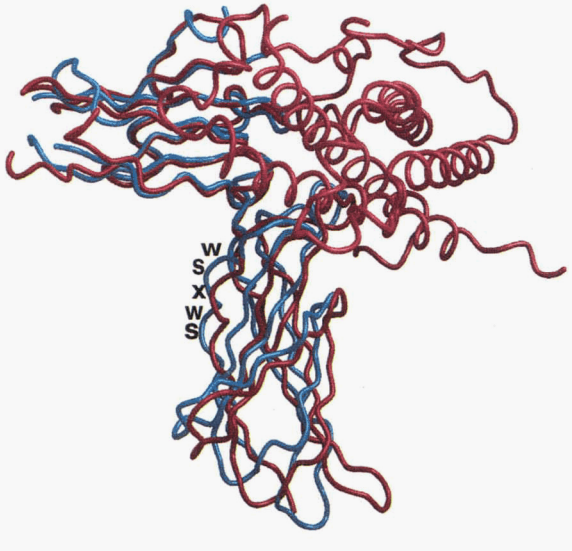

Fig. 2. Overlay of the intermediate 1:1 hGH–hGHR and hGH–hPRLR complexes. Only 1 copy of the hormone (in red) is shown (taken from the complex with hPRLR); the other copy superimposes with an RMS deviation in Cα positions of 0.8 Å. The hGHR is in blue and the hPRL receptor in red. Each receptor consists of 2 domains of about 110 residues each, connected by a 5-residue linker. In both receptors, some loops were not visible in the electron density maps and were omitted from the figure. The WSXWS box is labeled.

The hGH–hGHR and hGH–hPRLR 1:1 complexes have very similar overall structures (Fig. 2). Details of the structures of each of these complexes have been presented elsewhere, and thus only a summary will be given here. The structure of the hormone in these complexes is essentially the same and was the basis for the superposition in Figure 2. The overlay of the two 1:1 complexes shows that the surface interfaces between the hormone and each receptor are virtually identical. This is somewhat surprising because the sequence homology between the receptors is only 28%, and this percentage does not change significantly in the regions of the receptors making direct contact with the hormone. An obvious difference between the complexes exists in the positions of the C-terminal domains of the receptors. This difference involves a slight rotation of the C-terminal domain with respect to the N-terminal one, coupled with a translation of several Ångstroms. These differences are not thought to be artifacts of crystal packing but are important in forming the activated complex (see below).

Hormone and receptor binding sites for the 1:1 hGH–hGHR and hGH–hPRLR complexes

Binding site 1 on hGH, which is used in both hGH–hGHR and hGH–hPRLR complexes, is formed by residues that are exposed on helix 4 of the helix bundle, together with residues on the connecting loop between helix 1 and 2. The total surface area buried on the receptor is approximately 1,300 Å^2 (De Vos et al., 1992). Binding site 1 has a very distinct concave character. This contrasts to the much flatter surface at binding site 2. The packing of the 4 helices of hGH is very similar in both 1:1 complexes (RMS differences in Cα positions of 0.8 Å), indicating no global changes of the type seen in the analysis of the structure of an affinity-matured hGH mutant (Lowman & Wells, 1993; Ultsch et al., 1994). The largest differences in the hormone are in a small "mini-helix" of 2 turns (residues 38–47) in the segment connecting helices 1 and 2. The mini-helix differs by about 2.5 Å, depending to which of the 2 receptors it is bound; similar changes in this region were found in the affinity-matured variant (Ultsch et al., 1994).

The hormone binding surfaces of the receptors are formed by 6 closely spaced surface loops that extend from the β-sheet core in a manner somewhat similar to antigen binding loops in antibodies. Three loops, L1–L3, reside in the N-terminal domain; 2 others, L5 and L6, are found in the C-terminal domain. Binding loop L4 also serves as the linker between the domains. The conformations of these loops are very similar, as can be seen from the individually superimposed domains shown in Figure 3. However, in the intact receptors, the difference in the linker conformations reorient the respective domains, effectively changing the relative loop positions by up to 3.5 Å (Somers et al., 1994). Thus, comparing binding to site 1 of hGH between hPRLR and hGHR, global differences on the domain level are involved, leading to a rigid-body-like repositioning of the loops, which themselves have relatively conserved conformations. This con-

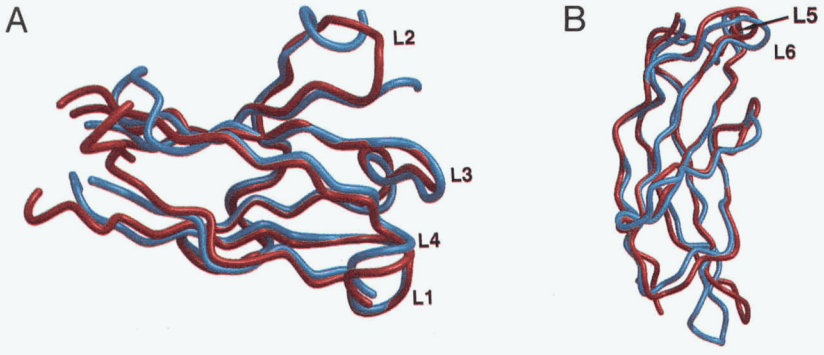

Fig. 3. Superposition of (**A**) the N-terminal and (**B**) the C-terminal domain of the hGHR (blue) and the PRLR (red). Receptor loops involved in ligand binding are labeled L1 through L6. Discontinuities represent disordered segments of the protein that were not included in the models.

trasts with the localized changes limited to the binding loops themselves, which are observed when the hGHR binds the 2 different sites (sites 1 and 2) on hGH in forming the 1:2 complex.

Comparison of functional and structural epitopes at binding site 1

The nature of the stereochemical factors determining the protein–protein recognition is just now emerging through a series of complementary mutagenesis and crystallographic studies (Cunningham & Wells, 1989, 1993; Bass et al., 1991; De Vos et al., 1992; A.M. De Vos & M. Ultsch, manuscript in prep.; T. Clackson & J.A. Wells, manuscript in prep.). Prior to the determination of the hGH–hGHR structure, an analysis of the residues on the hormone that most affected receptor site 1 binding was performed using alanine scanning mutagenesis (Cunningham & Wells, 1989); some additional interface residues identified from the structure were tested subsequently (Cunningham & Wells, 1993). The functional binding epitope was localized to a patch including residues from helix 4 and the loop connecting helices 1 and 2. In all, there were 13 residues that, when individually changed to alanine, produced at least a 1.5-fold decrease in receptor binding. Indeed, it was found that about 85% of the total binding energy was apparently developed by interactions of the receptor with just 8 residues on the hormone. In a separate study, a very similar picture emerged for site 1 in the hGH–hPRLR complex (Cunningham & Wells, 1991).

The structural epitope of hGH, as defined by those residues that directly contact the receptor, includes the so-called functional epitope but is considerably more extensive (De Vos et al., 1992; Cunningham & Wells, 1993). Thirty residues in hGH site 1 make contact with the receptor, corresponding to a 1,300-Å^2 surface area on the hormone. The 8 energy-contributing residues are sequestered in a surface patch that covers only about 32% (420 Å^2) of the total contact interface of hGH. There are no apparent distinguishing characteristics between the interactions that are energetically important from those of other side chains, which are energetically null. In the hGH–hGHR interface, there are 11 hydrogen bonds: 1 main-chain–main-chain, 2 main-chain–side-chain, and 8 side-chain–side-chain; only 5 of the 11 contribute significantly to the overall binding energy. A similar situation is observed in the analysis of the hGH–hPRLR site 1 interface (Somers et al., 1994). Four hGH side chains (K168, R167, D171, R178) form hydrogen bonds in both the hGHR and hPRLR complexes; however, only hGH K168 Nζ to receptor W104 O is conserved (receptor residues are numbered based on the hGHR).

Conformational differences in the receptor binding loops result in some residues (like R167) of hGH forming hydrogen bonds to different partners in the respective receptor complexes (see below), whereas some other hydrogen bonds are eliminated altogether (for example hGH R178 to hGHR E120). In addition, the sequence and conformational differences produce differences in the local environment, altering the relative effective energies of the hydrogen bonds between the 2 systems. For example, mutation of R64 of hGH to alanine caused a large decrease in affinity for the GHR (Cunningham & Wells, 1989, 1993) but had no effect on the affinity for the PRLR (Cunningham & Wells, 1991). The crystal structures show that, in the hGH–hGHR complex, a salt bridge interaction is made between R64 and D164 of the receptor, but in the hGH–hPRLR complex, the Cα of 164 has moved by about 3.5 Å, placing the side chain far from the guanidinium of 64 and greatly diminishing the electrostatic interaction between the 2 groups. Thus, the difference in importance for binding of the 64 side chain in the 2 complexes can be assigned directly to hydrogen bonding effects.

The example of R167 shows that the presence of an intermolecular hydrogen bond, even a charged one, does not necessarily contribute to binding energy. In the hGH–hGHR complex, the side chain of R167 forms a good hydrogen bond with E127 of the receptor (2.9 Å) (De Vos et al., 1992). Surprisingly, this interaction had been shown by the alanine-scanning mutagenesis to have little influence on binding (Cunningham & Wells, 1989). In the hGH–hPRLR complex, a similar salt bridge exists between R167 and D124 of the hPRLR (Somers et al., 1994); however, in this case, the interaction is an important contributor to the binding energy of the complex (Cunningham & Wells, 1991). The comparison of the 2 structures provides insight into why R167 of hGH contributes energetically to stability of 1 complex, but not the other. In the case of the hGH–hGHR complex, the side chains participating in the hydrogen bond are not involved in additional interactions. In contrast, in the hGH–hPRLR complex, the carboxylate position of D124 is stabilized in the receptor through an internal hydrogen bond to T106 Oγ1 (2.6 Å). Moreover, the arginine side chain is sandwiched between 2 tyrosine side chains, Y28 from the hormone and Y127 from the receptor (Fig. 4). This situation is an example of the importance of entropic and desolvation factors of the unbound states for protein–protein association (Ross & Subramanian, 1981; Hintz, 1983). In the hGH–hGHR complex, the enthalpy gained by the interaction between R167 and E127 is offset by a loss in side-chain entropy compared to the unbound state. On the other

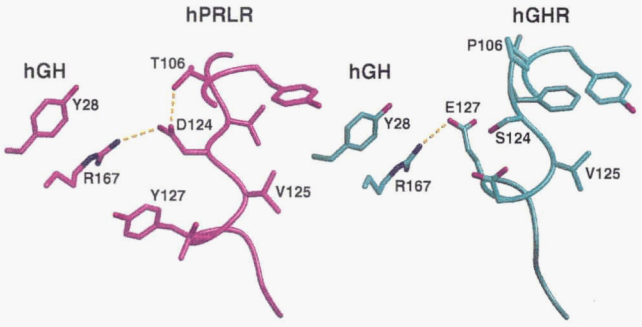

Fig. 4. Interactions of R167 of hGH with the PRLR (in magenta; left-hand side) and with the hGHR (in cyan; right-hand side). Because of rigid-body changes in the disposition of the respective receptors, the arginine interacts with nonequivalent receptor side chains: in the hPRLR, with position 124 (Asp), but in the hGHR, with position 127 (Glu). Note that in the hGH–hPRLR complex, but not in the hGH–hGHR complex, the arginine side chain is sandwiched between 2 aromatic side chains. (Amino acid numbering for the receptors is based on the hGHR, so that structurally equivalent residues have identical residue numbers.)

hand, in the hPRLR, the carboxylate of D124 is already held in position by T106, so that the entropic cost of stabilizing its position has already been paid; in addition, the hydrogen bond is shielded from solvent by the side chain of Y127.

Mechanism of the transition between the intermediate 1:1 to the active 1:2 complex

An important question concerning the mechanism of association of these receptors is whether there is an obligatory conformational change that occurs going from the intermediate 1:1 complex to the active 1:2 form, or whether the 1:1 complex is essentially a preformed template onto which the second receptor can bind without any adjustment. Based on the structure of the 1:2 hGH–hGHR complex, an important element to forming the stable complex is the extensive interaction (500 Å^2) between the 2 hGH receptors along a β-sheet surface in the C-terminal domain. This interface is characterized by a highly structured set of hydrogen bonding and van der Waals interactions (Fig. 5A).

For the formation of active hGH–hGHR complex, there is direct evidence that the 1:1 complex presents a preformed template. De Vos and Ultsch (manuscript in prep.) showed that the independently determined structure of the intermediate complex could be directly superimposed on the structures of the hormone and receptor 1 in the 1:2 complex, with an RMS deviation of 0.9 Å in Cα positions. The magnitude of these deviations indicates that no substantial differences exist between the disposition of the hormone to receptor 1 in these complexes. Unfortunately, no similar comparison can be made for the hGH–hPRLR system because only the structure of the 1:1 complex is available.

As shown in Figure 2, there are some clear differences in the structures of the 1:1 complexes, especially at the C-terminal base of the receptors. This is the same region where extensive contacts are made between the 2 receptor molecules in the 1:2 ternary complex in hGH–hGHR (De Vos et al., 1992). Additionally, there is no sequence homology between the 2 receptors at the expected receptor–receptor interfaces. These factors indicate that there will be differences at the receptor interfaces in the hPRL system to make a competent ternary complex. These differences raise the issue as to how comparable the mechanism of binding of the second receptor is between the 2 systems.

To gain some insight into whether the hGH–hPRLR structure is a preformed template for binding the second receptor, as is the case for the hGH–hGHR 1:1 complex, 2 simple modeling experiments were performed. First, using the 1:2 hGH–hGHR complex as a model, hPRLR was superimposed on both hGHRs at sites 1 and 2; this simply substituted 2 hPRLR receptors for the 2 hGHR counterparts. This model was clearly not feasible without additional conformational changes because of steric clashes that occurred at the C-terminal base of the receptors.

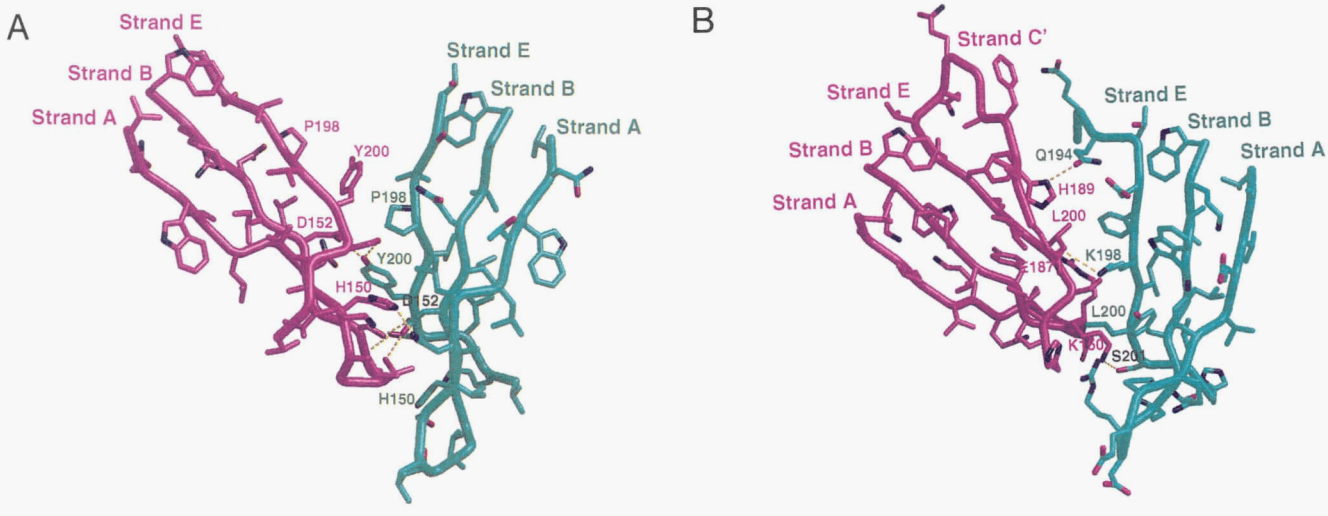

Fig. 5. Interactions between (**A**) the hGHRs and (**B**) the hPRLRs along the β-sheet stems of their C-terminal domains. The interactions in the case of the hGH receptors were observed in the 1:2 hGH–hGHR complex (De Vos et al., 1992). The interaction between surfaces of the hPRL receptors was developed through model building based on the 1:1 hGH–hPRLR complex (Somers et al., 1994).

In the second modeling exercise, the hGH–hPRLR complex was taken as determined from the X-ray structure. The putative 1:2 complex was built by placing a second hPRLR molecule in binding site 2, in a manner identical to that observed in the hGH–hGHR 1:2 complex. In contrast to the first experiment, this arrangement produces receptor–receptor contact points that show remarkable complementarity between the 2 molecules, even to the level of seeing potential intermolecular hydrogen bonds in the interfaces (Fig. 5B). The principal residues involved are K150, E187, H189, and S201 on 1 receptor and Q194, F197, K198, and S201 on the other receptor face. In both the hGHR and hPRLR 1:2 complexes, S201 appears to play an important role in crosslinking the receptor trunks by forming a set of 2 hydrogen bonds across the interface. Otherwise, most of the points of contact differ between the 2 receptors. In the hGHR interface, there is a concentration of contacts involving several residues in strand B (between residues 143 and 152). In hPRLR, most of the contact points are on strands C′ and E (residues 189–201).

We conclude that this modeling exercise makes a compelling case for the premise that the observed differences in the dispositions of hGH to the 2 receptors are not an artifact of crystal packing but real and functional. It would, therefore, follow that the mechanism of second receptor binding is identical in the 2 systems: the 1:1 complexes exist as preformed templates to assist the binding of the second receptor.

The WSXWS box

The most highly conserved sequence in class 1 of the hematopoietic receptor superfamily is the pentapeptide Trp-Ser-[any amino acid]-Trp-Ser near the C-terminal end of the ligand-binding domain (see Fig. 1). The functional significance of this motif has been debated, and it has been proposed to be critical for receptor activity either because it is involved in ligand binding, or in interactions with a putative accessory protein (Patthy, 1990; Rozakis-Adcock & Kelly, 1992), or because it is critical for the proper folding of the C-terminal domain. Attempts to define the function of these residues by mutagenesis studies have confirmed the overall importance of this motif for signal transduction but have been unsuccessful in providing a more detailed answer (Miyazaki et al., 1991; Chiba et al., 1992; Rozakis-Adcock & Kelly, 1992).

The 3-dimensional structure of the prolactin receptor shows that the WSXWS box is located shortly before strand G of the 4-stranded sheet of the C-terminal domain (Somers et al., 1994). The outside surface of this sheet remains solvent exposed in the active 1:2 complex. The main chain of the Trp-Ser-Ala-Trp-Ser sequence is in an irregular extended conformation but does not form a regular pattern of hydrogen bonds to neighboring strand F (Fig. 6). The side chains of the tryptophans as well as the alanine are disposed at the solvent-accessible surface of the sheet while the serines point inside, the Oγ atom hydrogen bonding to the carbonyl oxygen of the following residue and to a main-chain amide of strand F. There is a pronounced bulge in the main chain at the position of the alanine. Strand G does not begin immediately after the residues making up the WSXWS box. Following the motif is the sequence Pro-Ala-Thr. The proline forms a main-chain bulge very similar to that seen at the central residue of the WSXWS box, and regular main-chain hydrogen bonds to the neighboring strand start only with the threonine.

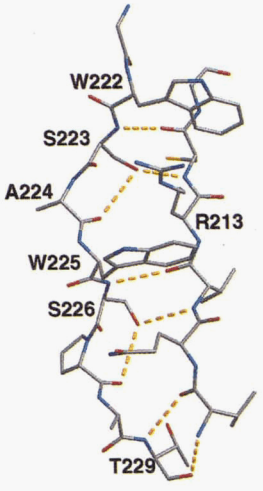

Fig. 6. Main-chain conformation of the segment containing the WSXWS box in the hPRLR, including interactions with the neighboring β-strand, F. Observed hydrogen bonds are shown as yellow dotted lines. Because of bulges at A224 and P227, strand G begins at T229.

To answer the question whether the observed main-chain conformation is characteristic of the WSXWS box sequence, we can compare the local structure of this segment to that of the equivalent regions in other fibronectin type III modules whose structures are known. The equivalent sequence in the hGHR is Tyr-Gly-Glu-Phe-Ser, followed by Asp-Ser. The main-chain conformation of this segment is identical to that of the PRLR, including the 2 bulges and the disposition of the side chains; a water molecule occupies the precise position of the Oγ of the first serine of the motif in the PRLR (A.M. De Vos & M. Ultsch, manuscript in prep.). Tissue factor, a member of class 1 of the superfamily, has the sequence Lys-Ser-Thr-Asp-Ser, followed by Pro-Val, and even though in this case the aromatic residues are not conserved, the conformation of this segment is again the same as in the PRLRs and GHRs (Muller et al., 1994). The same observation holds for the structure of the first of the tandem fibronectin domains from neuroglian (Pro-Ser-Ala-His-Ser-Asp-Ser), but not for the second of these domains where a regular stretch of β-strand is found (Huber et al., 1994). Finally, in the third domain of tenascin, where the sequence is Ser-Ser-Asn-Pro-Ala, the main chain starts out similarly but achieves regular β-strand character from the alanine onward: the first bulge at the asparagine is present, but the second bulge is absent and there is instead a deletion with respect to the cases described above (Leahy et al., 1992). From this survey, we conclude that the tryptophans are not important for the observed local main-chain conformation, and even though the main chain is likely to have a conserved conformation for all class 2 receptors, this conformation is not unique to these cases and probably not sufficient to support a specific biological role.

The picture changes dramatically, however, when the larger surroundings of the WSXWS box are taken into consideration (Fig. 7). In the cases of the GHRs and PRLRs, the surface of the 4-stranded sheet forms a patch containing a striking pattern of pairs of charged or hydrophilic side chains, coming from strands C and F, interleaved by the side chains of the aromatic residues of the WSXWS box (Somers et al., 1994). The net re-

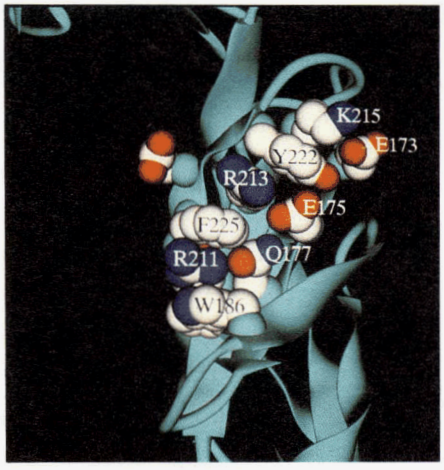

Fig. 7. Surface of the 4-stranded sheet of the C-terminal domain of the hGHR, showing the large motif surrounding the WSXWS box. In the hGHR, the residues corresponding to the tryptophans are Y222 and F225; their side chains intercalate between the hydrophobic parts of the long, charged side chains of the neighboring strand. The resulting surface displays many functional groups into the solvent. A similar pattern is observed in the hPRLR (Somers et al., 1994).

sult is a highly charged, solvent-exposed surface, which is tightly packed as a result of the intimate interactions between aromatic groups and the side-chain methylenes of the charged and hydrophilic side chains. In the case of the PRLR, a series of 3 spatially successive arginine residues combines with the exposed nitrogen atoms of the indole rings to create a repeating pattern of positive charges. For the GHR, a similar pattern can be observed, though in this case two of the positive charges are compensated by neighboring glutamates (Fig. 7) (De Vos et al., 1992). Inspection of a sequence alignment of other hematopoietic receptors containing the WSXWS box (Bazan, 1990) demonstrates that equivalent patterns can be expected in these cases; indeed, a recent model of the interleukin-4 receptor displays a similar patch (A. Gustchina, pers. comm.). From a structural perspective, the tight packing of the side chains displayed on this sheet would likely contribute to the stability of the β-sandwich. However, the large number of functional groups covering this surface certainly makes it an attractive candidate for protein–protein interactions. Thus, it could certainly represent an interactive surface for as yet unidentified accessory proteins.

Tissue factor: A member of class 2 of the hematopoietic receptor superfamily

Class 2 of the hematopoietic receptor superfamily consists of the receptors for α- and γ-interferon together with tissue factor (Bazan, 1990). Tissue factor is the exception in the superfamily because it is not a true signaling receptor. Instead, it is a membrane-bound protein functioning as the protein cofactor for factor VIIa. The tissue factor–factor VIIa complex initiates the extrinsic pathway of blood coagulation by activating factor X, ultimately leading to fibrin deposition and clot formation.

Tissue factor has an extracellular domain of 219 residues, a short, 23-residue transmembrane segment, and a small cytoplasmic domain (residues 243–263). Determination of the 3-dimensional structure of the extracellular portion (Muller et al., 1994) has confirmed its classification in the hematopoietic superfamily: it consists of 2 domains of approximately equal size, connected by a single short linker segment (Fig. 8). In tissue factor, both domains have standard fibronectin type III topology: a sandwich containing 2 β-sheets, one containing strands A, B, and E, the other C, C′, F, and G. This differs subtly from the GHRs and PRLRs, where strand C′ in the N-terminal domain is broken into 2 short pieces, one hydrogen bonding to strand C of the second sheet, the other to strand E of the first. A more obvious difference from the receptors from class 1 is the relative orientation of the 2 domains: in tissue factor, the "elbow angle" between them is about 120° instead of 85° (Fig. 8). This difference has a dramatic effect on the domain–domain interface region, and some of the loops that were solvent exposed

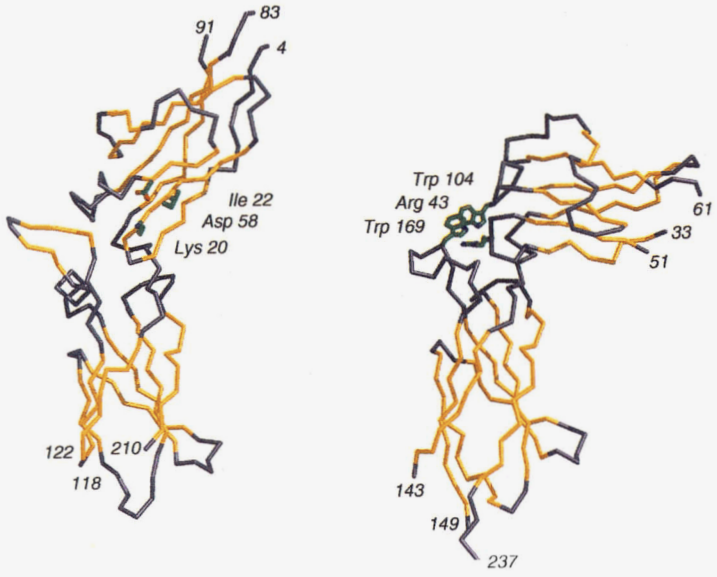

Fig. 8. Backbone structure of tissue factor (left) and the hGHR (right). β-Strands are colored yellow. Loops not seen in the electron density map are left out and chain ends are labeled. Residues in green are the most important binding determinants: in tissue factor, K20, I22, and D58 for factor VII binding (Muller et al., 1994); in the hGHR, R43, W104, and W169 for hGH binding (De Vos et al., 1992; T. Clackson & J.A. Wells, manuscript in prep.).

in the GHRs and PRLRs are largely buried between the domains in tissue factor. A noteworthy example is the AB loop: R43 of the GHR is an important binding determinant and forms hydrogen bonds with the ligand, whereas F19, the homologous residue of tissue factor, is completely buried in the center of the domain–domain interface. Interestingly, the change in orientation is not accompanied by a different conformation of the domain–domain linker: in tissue factor, as well as the receptors of class 1, the linker is helical. This is in contrast to the connection between the 2 fibronectin type III domains of neuroglian, which has an extended conformation.

The ligand-binding determinants of the GHRs and PRLRs are localized at the outside of the elbow at the domain–domain interface. Therefore, the change in environment of the residues in this region created by the differences in domain–domain orientation and interface has important implications for the binding epitope for factor VII on tissue factor. Mutagenesis has identified 3 major binding determinants in tissue factor: K20, I22, and D58. When mapped on the 3-dimensional structure, these residues are found near the domain–domain interface but at the inside of the elbow (Fig. 8). Thus, the binding site has "migrated" to the opposite side of the molecule.

It is interesting to consider the relevance of the tissue factor example for the other receptors in class 2 of the superfamily. Do the sequence similarities among these receptors mean that they have a similar domain–domain orientation as tissue factor, and are their ligand binding sites located at the inside of the elbow rather than at the outside? Or does the overall structure of the ligand determine the conservation of the location of the binding site? In that case, tissue factor would be the exception because factor VII is a multidomain protein, whereas the ligands for the cytokine receptors are all helical bundles. In the present state of our knowledge, it is perhaps best not to speculate but to await a crystal structure of a class 2 ligand–receptor complex.

Concluding remarks

The binding of the endocrine hormones to their respective receptors is a highly regulated process. In primate biology, hGH is unique in its ability to bind and activate the PRLR, whereas PRL does not bind to the GH receptor. Human placental lactogen (hPL) activates the PRLR, although the hormones have only limited sequence homology. However, hPL does not bind the hGH receptor, even though it has 85% sequence identity to hGH. Growth hormones are highly conserved, so it is not unexpected that hGH activates the GH receptors of other species; however, it is unclear why the converse is not operative: other hormones cannot activate the hGHR (Nicoll et al., 1986). Taken together, these observations present somewhat of a puzzle. On the one hand, upon formation of the active 1:2 complex the GHRs and PRLRs are versatile enough to complement highly diverse topographic surfaces at the 2 sites on the hormone. On the other hand, specificity is apparently dictated by relatively few key residues because molecules such as hPL or GH from other species, highly similar to hGH, do not bind the hGHR.

Although the various structures of hormone–receptor complexes provide information that encompasses both the versatility and specificity components inherent in the recognition system that regulates endocrine biology, a general understanding of this process at the molecular level is difficult to construct, even combining it with the extensive mutational database available to us. One is struck by the extraordinary adaptability of these molecules to synthesize competent binding epitopes to a wide range of large target surfaces. The nature of the adjustments required to form the optimum set of interactions between the hormone and each of its 2 receptors suggests that recognition and binding of the 2 protein surfaces is directed by an induced-fit mechanism. Distinct from the process of molecular recognition associated with the antibody–antigen paradigm, where binding is developed mainly through sequence diversity of the antibody complementarity-determining loops, the endocrine receptors use essentially a constant set of residues to bind surfaces that are diverse both in sequence and in conformation. This is accomplished by employing conformational diversity, both local and global.

The binding surfaces between the hormones and receptors are substantial, significantly larger than those associated with antibody–antigen interfaces. With these extensive contact surfaces, how can molecules on the one hand show such binding versatility, yet on the other display the finely honed specificity required for biological regulation? The answer to this question appears to be that the binding and specificity determinants are generally different and involve a small fraction of the residues in the contacting interface. In fact, it has been demonstrated that binding specificity can be almost totally altered by mutating a few key residues (Cunningham & Wells, 1991). Although the structures of the receptor complexes discussed here represent a rich database for beginning to develop an understanding of the trends governing molecular recognition, we feel that to establish general rules (if they exist), additional structural information based on the mutational analyses is required. This work is currently in progress.

Acknowledgments

We thank Drs. Tim Clackson and Jim Wells for providing unpublished data.

References

Abdel-Meguid SS, Shieh HS, Smith WW, Dayringer HE, Violand BN, Bentle A. 1987. Three-dimensional structure of a genetically engineered variant of porcine growth hormone. *Proc Natl Acad Sci USA 84*:6434–6437.

Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, Loetscher H, Lesslauer W. 1993. Crystal structure of the soluble human 55 kd TNF receptor–human TNFβ complex: Implications for TNF receptor activation. *Cell 73*:431–445.

Bass SH, Mulkerrin MM, Wells JA. 1991. A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. *Proc Natl Acad Sci USA 88*:4498–4502.

Baumann G, Lowman HB, Mercado M, Wells JA. 1994. The stoichiometry of growth hormone-binding protein complexes in human plasma: Comparison with cell surface receptors. *J Clin Endocrinol Metab 78*:1113–1118.

Bazan JF. 1990. Structural design of molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA 87*:6934–6938.

Boulay JL, Paul WE. 1993. Hematopoietin sub-family classification based on size, gene organization and sequence homology. *Curr Biol 3*:573–581.

Chawla RK, Parks JS, Rudman D. 1983. Structural variants of human growth hormone: Biochemical, genetic and clinical aspects. *Annu Rev Med 34*:519–547.

Chiba T, Amanuma H, Tokodoro K. 1992. Tryptophan residue of Trp-Ser-X-Trp-Ser motif in extracellular domains of erythropoietin receptor is essential for signal transduction. *Biochem Biophys Res Commun 184*:485–490.

Cosman D. 1993. The hematopoietin receptor superfamily. *Cytokine 5*: 95–106.

Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, Wells JA. 1991. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science 254*:821–825.

Cunningham BC, Wells JA. 1989. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science 244*: 1081–1085.

Cunningham BC, Wells JA. 1991. Rational design of receptor-specific variants of human growth hormone. *Proc Natl Acad Sci USA 88*:3407–3411.

Cunningham BC, Wells JA. 1993. Comparison of a structural and a functional epitope. *J Mol Biol 234*:554–563.

De Vos AM, Kossiakoff AA. 1992. Receptor action and interaction. *Curr Opin Struct Biol 2*:852–858.

De Vos AM, Ultsch M, Kossiakoff AA. 1992. Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. *Science 255*:306–312.

Fuh G, Colosi P, Wood WI, Wells JA. 1993. Mechanism-based design of prolactin receptor antagonists. *J Biol Chem 268*:5376–5381.

Hintz HJ. 1983. Thermodynamics of protein-ligand interactions: Calorimetric approaches. *Annu Rev Biophys Bioeng 12*:285–317.

Huber AH, Wang YM, Bieber AJ, Bjorkman PJ. 1994. Crystal structure of tandem type III fibronectin domains from *Drosophila* neuroglian at 2.0 Å. *Neuron 12*:717–731.

Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvennoinen O. 1994. Signaling by the cytokine receptor superfamily: JAKs and STATSs. *Trends Biochem Sci 19*:222–227.

Kaushandy K, Karplus PA. 1993. Hematopoietic growth factors: Understanding functional diversity in structural terms. *Blood 82*:3229–3241.

Leahy DJ, Hendrickson WA, Aukhil I, Erickson HP. 1992. Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. *Science 257*:987–991.

Lowman HB, Wells JA. 1993. Affinity maturation of human growth hormone by monovalent phage display. *J Mol Biol 234*:564–578.

Miyazaki T, Maruyama M, Yamada G, Hatakeyama M, Taniguchi T. 1991. The integrity of the conserved WS motif common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction. *EMBO J 10*:3191–3197.

Müller YA, Ultsch M, Kelley RF, De Vos AM. 1994. Structure of the extracellular domain of tissue factor: Location of the factor VIIa binding site. *Biochemistry*. Forthcoming.

Nicoll CS, Mayer GL, Russell SM. 1986. Structural features of prolactins and growth hormones that can be related to their biological properties. *Endocrine Rev 7*:169–203.

Patthy L. 1990. Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. *Cell 61*:13–14.

Ross PD, Subramanian S. 1981. Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry 20*:3096–3107.

Rozakis-Adcock M, Kelly PA. 1992. Identification of ligand binding determinants of the prolactin receptor. *J Biol Chem 267*:7428–7433.

Somers W, Ultsch M, De Vos AM, Kossiakoff AA. 1994. The X-ray structure of the growth hormone-prolactin receptor complex: Receptor binding specificity developed through conformational variability. *Nature*. Forthcoming.

Sprang SR, Bazan JF. 1993. Cytokine structural taxonomy and mechanisms of receptor engagement. *Curr Opin Struct Biol 3*:815–827.

Ultsch M, De Vos AM. 1993. Crystals of human growth hormone-receptor complexes. Extracellular domains of the growth hormone and prolactin receptors and a hormone mutant designed to prevent receptor dimerization. *J Mol Biol 231*:1133–1136.

Ultsch M, Somers W, Kossiakoff AA, De Vos AM. 1994. The crystal structure of affinity-matured human growth hormone at 2 Å resolution. *J Mol Biol 236*:286–299.

Wells JA, De Vos AM. 1993. Structure and function of human growth hormone: Implications for the hematopoietins. *Annu Rev Biophys Biomol Struct 22*:329–351.