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Receptor binding properties of four-helix-bundle growth factors deduced from electrostatic analysis

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Abstract

Hormones of the hematopoietin class mediate signal transduction by binding to specific transmembrane receptors. Structural data show that the human growth hormone (hGH) forms a complex with a homodimeric receptor and that hGH is a member of a class of hematopoietins possessing an antiparallel 4-α-helix bundle fold. Mutagenesis experiments suggest that electrostatic interactions may have an important influence on hormone–receptor recognition. In order to examine the specificity of hormone–receptor complexation, an analysis was made of the electrostatic potentials of hGH, interleukin-2 (IL-2), interleukin-4 (IL-4), granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), and the hGH and IL-4 receptors.

The binding surfaces of hGH and its receptor, and of IL-4 and its receptor, show complementary electrostatic potentials. The potentials of the hGH and its receptor display approximately 2-fold rotational symmetry because the receptor subunits are identical. In contrast, the potentials of GM-CSF and IL-2 lack such symmetry, consistent with their known high affinity for hetero-oligomeric receptors. Analysis of the electrostatic potentials supports a recently proposed hetero-oligomeric model for a high-affinity IL-4 receptor and suggests a possible new receptor binding mode for G-CSF; it also provides valuable information for guiding structural and mutagenesis studies of signal-transducing proteins and their receptors.

Keywords: cytokines; electrostatic potential; hematopoietic receptors; human growth factor; interleukins; molecular recognition

Electrostatic forces play an important part in molecular recognition. By acting at much greater distances than other molecular forces, they provide a driving force for the proper docking of ligands into their protein binding sites and for protein–protein association. Such electrostatic steering has been shown for the enzyme–substrate encounter of superoxide dismutase (Sines et al., 1990), triosephosphate isomerase (Luty et al., 1993; Wade et al., 1994), and acetylcholinesterase (Ripoll et al., 1993), and for the protein–protein encounter in the formation of the cytochrome *c*/cytochrome *c* peroxidase complex (Northrup et al., 1988), the complex of plastocyanin with cytochrome *c* (Roberts et al., 1991), and a lysozyme–antibody complex (Kozack & Subramaniam, 1993). Electrostatic forces contribute to the specificity of protein complexation (Perry et al., 1989), which often involves complementary charge distributions on the binding faces, such as have been observed for the thrombin–hirudin complex (Karshikov et al., 1992).

Indications that electrostatic properties influence both the diffusional association and the binding affinity of growth factors to their receptors are provided by mutagenesis studies (Cunningham & Wells, 1993). For example, the mutation of Glu 9 in IL-4 to lysine results in a 1,400-fold drop in receptor binding affinity (Kruse et al., 1993). Formation of a specific high-affinity hormone–receptor complex is likely to be dependent on formation of complementary van der Waals and electrostatic interactions. In this paper, we investigate the electrostatic properties of these hormones and their receptors in order to examine their contribution to the specificity of these interactions.

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Abbreviations: hGH, human growth factor; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; hG-CSF, human granulocyte colony-stimulating factor; bG-CSF, bovine granulocyte colony-stimulating factor; IL, interleukin; IL-2Rγ, interleukin-2 receptor γ chain; IL-4R, interleukin-4 receptor chain; Ig, immunoglobulin; PB, Poisson-Boltzmann; RMSD, RMS deviation.

A large group of hormones known as growth factors regulate the course of cell growth and differentiation by binding to specific receptors embedded in the cell membrane (Bazan, 1990; McDonald & Hendrickson, 1993). These hormones transmit cell signals by inducing the oligomerization of receptor molecules (Cunningham et al., 1991; Boulay & Paul, 1992; de Vos et al., 1992; Miyajima et al., 1992; Foxwell & Barrett, 1993; McDonald & Hendrickson, 1993). Depending on the binding properties of the hormone, its receptor may consist of identical or different receptor molecules, hence forming homo- or hetero-receptor-subunit hormone–receptor complexes. Most hormone–receptor complexes are thought to consist of 3 polypeptide chains: 1 of the hormone and 2 of the receptor (Miyajima et al., 1992). However, a third hormone binding receptor subunit of the IL-2 receptor has been found and a quaternary model has been proposed for the hormone–receptor complex (Minami et al., 1993).

Here we focus on the 4-α-helix bundle class of growth factors (McDonald & Hendrickson, 1993). This includes, among others, hGH and the hematopoietic cytokines (hematopoietins): M-CSF, G-CSF, GM-CSF, IL-2, IL-4, and IL-5. These hormones are relatively small (10–30 kDa) glycoproteins stabilized by disulfide bonds. With the exception of M-CSF and IL-5, which are disulfide-linked homodimers, all other known hematopoietic hormones are single subunit proteins. They display very little sequence similarity. However, crystallographic and NMR studies show that most of these hormones share a well-preserved common 3D fold. This is characterized as a left-twisted 4-α-helix bundle with the helices connected by an up-up-down-down topology (Fig. 1; Kinemage 1). The helices are less parallel than those in classical 4-helix bundles (Smith et al., 1992), having interhelix angles of more than 40°. This type of geometry and topology is unique among 4-helix bundle proteins and, hence, cytokines can be distinguished as a distinct structural class of proteins (Hill et al., 1993). However, the individual cytokine structures differ in their details, particularly in the relative lengths of the bundle-forming helices.

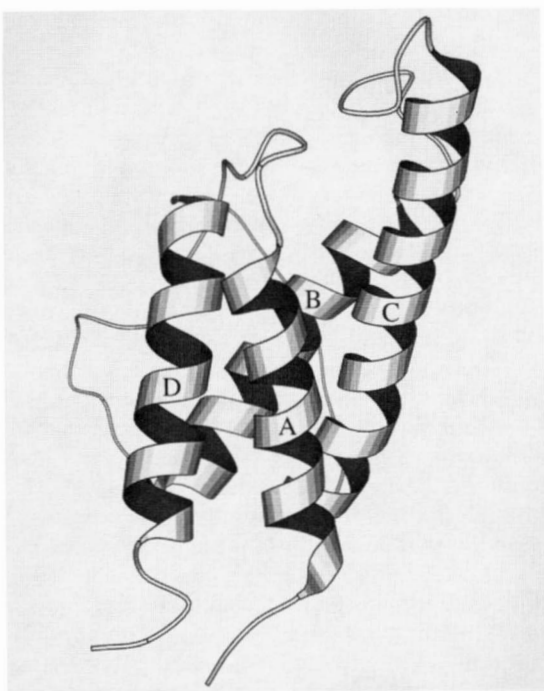

Fig. 1. Ribbon diagram of an NMR structure of interleukin-4 (Müller et al., 1994). The helix bundle has up-up-down-down connectivity. The 4 major α-helices are labeled A (residues 4–20), B (39–58), C (69–95), and D (108–125). The picture was prepared using the MOLSCRIPT program (Kraulis, 1991) according to DSSP (Kabsch & Sander, 1983) secondary structure assignments.

Functional hematopoietic receptors are transmembrane proteins consisting of several components, including an extracellular ligand binding part and transducer, effector, and regulatory subunits (Miyajima et al., 1992). In contrast to the hematopoietins, the sequences of the extracellular segments of the receptors are related. They constitute the hematopoietin receptor superfamily (Cosman et al., 1990; Bazan, 1993). Only one 3D structure of a receptor has been published to date: the structure of the ternary complex of hGH and the extracellular segments of 2 identical receptor molecules, which has been solved by X-ray crystallography (de Vos et al., 1992). The structure of each receptor unit is similar to that of the extracellular regions of rat CD2 (Jones et al., 1992) and human CD4 (Wang et al., 1990), which belong to a class of immunoglobulin molecules known to be responsible for many cellular recognition processes.

Most hormone–receptor interactions are specific. There is little cross-reactivity between hormones and receptors of different species or different types, unless (1) both are highly homologous (Fukunaga et al., 1991; Nicola & Metcalf, 1991), or (2) the hormones share a common receptor component (Boulay & Paul, 1992; Kondo et al., 1993; Russell et al., 1993), or (3) have different binding sites on the hormone (Fuh et al., 1993). An indication that a part of the specificity of hormone–receptor recognition may be dependent on electrostatic interactions is given by observations that mutations involving charged residues in IL-2 (Weigel et al., 1989; Zurawski et al., 1990; Zurawski & Zurawski, 1992), IL-4 (Kruse et al., 1992, 1993; Ramanathan et al., 1993; Wlodawer et al., 1993), GM-CSF (Kaushansky et al., 1989; Lopez et al., 1992), and hGH (Cunningham et al., 1991; Cunningham & Wells, 1993; Wells & de Vos, 1993) and its receptor (Bass et al., 1991) often have a significant effect on receptor binding. We have therefore carried out a comparative study of the electrostatic properties of 5 growth factors, the extracellular part of the hGH receptor, and a homology-modeled extracellular part of the 130-kDa chain (IL-4R) of the IL-4 receptor in order to examine their binding modes.

Electrostatic potentials were calculated by numerical solution of the linearized finite-difference Poisson–Boltzmann equation using a macroscopic continuum-dielectric model of the protein in ionic solvent (Davis & McCammon, 1989). In this model, (1) partial charges are assigned to protein atoms, (2) the protein is treated as a low dielectric cavity inserted in a high dielectric solvent medium with the dielectric boundary determined by the 3D structure of the protein, and (3) ions are assumed to be distributed around the protein according to the Boltzmann distribution.

Comparison of the electrostatic potentials of hGH, hG-CSF, bG-CSF, GM-CSF, IL-2 and IL-4, the hGH/hGH-receptor complex, and a modeled complex of IL-4/IL-4R shows that the specificity of hormone–receptor binding is related to the distribution of the electrostatic potential around the hormone. In particular, the electrostatic potential indicates whether a hormone stimulates the formation of homodimeric or hetero-oligomeric receptor complexes.

Results

Complementarity of electrostatic potentials

An example of the complementarity of the electrostatic potentials is given by hGH, which forms a high-affinity complex with 2 hGHR molecules despite the fact that both the hGH and the extracellular segment of the receptor are negatively charged. Assuming standard amino acid residue protonation states at pH 7, the net charge on the hormone is $-4e$ (measured pI = 5.0; Gellerfors et al., 1989) and the net charge on the extracellular part of the receptor dimer is $-14e$. The electrostatic potentials of the interacting proteins are, however, distributed so that there are regions of complementary positive and negative potential at the hormone–receptor interface (Fig. 2). Six regions of complementarity in the electrostatic potentials of the receptor and the hormone are distinguishable at the interface region. These are indicated in Figure 2 by letters **a**–**f** in the hormone and by letters **a′**–**f′** in the receptor dimer (see also Fig. 9A for the hormone).

We identified the charged residues responsible for the observed electrostatic potential pattern and compared them to those listed in Table 2 of de Vos et al. (1992). In addition to the residues making the 5 salt bridges listed in that table, other residues were found that make favorable intermolecular electrostatic interactions although these do not necessarily contact each other. The positively charged region **a′**, located at the bottom of the hormone-binding groove (Fig. 2A), is in the center of the hormone–receptor interface (Fig. 2B). It is formed by Arg 43 and Arg 217 of the receptor *A*-chain and residues Arg 43, Lys 167, and Arg 217 of the receptor *B*-chain. In the hormone–receptor complex, these residues interact with Asp 11, Asp 26, Asp 171, and Glu 174 (negative region **a**) of hGH. The **a′** region of the receptor potential is surrounded by negative potential. Regions **b′** and **c′**, formed by residues Glu 42, Glu 44, and Asp 164 of the receptor *A*- and *B*-chains, respectively, have negative potential. They interact with residues Arg 64, Arg 178, and Arg 183 (region **b**) and Arg 16 and Arg 19 (region **c**) of hGH. Regions **d′** and **e′** (residues Asp 126 and Glu 127 of the receptor *A*- and *B*-chains, respectively) are 2 further regions of negative receptor potential adjacent to the center of the hormone binding interface. They interact with 2 corresponding regions of positive potential on hGH: region **d** (residues Lys 38, Lys 41, Arg 167, Lys 168, and Lys 172) and region **e** (residues Arg 8 and Arg 127). A peripheral α-helix located between the A and B helices of the hormone (residues Lys 38–Gln 46) contributes to the negative region **f** (residues Glu 39 and Asp 154) in the electrostatic potential of hGH, which contacts the positively charged region **f′** of the receptor (residues Lys 34, Lys 110, and Lys 121 of the *A*-chain).[3] Thus, the electrostatic potentials around the binding faces of the hGH and its receptor seem to be balanced to promote a complementary recognition.

A quantitative estimate of electrostatic complementarity between hormone and receptor molecules was made by calculating the electrostatic interaction energy between the hormone and a receptor subunit as a function of their mutual orientation using the test charge approximation (see Equation 1). Figure 3 shows an energy surface obtained for the receptor *A*-chain on rotating and translating it in the electrostatic potential of the hormone. There are 2 favorable low-energy orientations for the receptor *A*-chain: one at about 160° and the other at about 350°. They correspond to the orientations of the *A*- and *B*-chains of the receptor in the ternary complex[4] and indicate regions of complementary electrostatic potential.

Another example of hormone–receptor electrostatic potential complementarity is given by the modeled IL-4/IL-4-receptor complex (Fig. 4). Bamborough et al. (1993) have modeled the complex by homology on the basis of the CD4 coordinate set assuming that the functional IL-4 receptor consists of 2 identical IL-4R chains. However, we have experimentally identified only 1 binding site for the IL-4R on the IL-4 molecule (Kruse et al., 1993). It is formed by residues of the A and C helices and binds the receptor *B*-chain of the model (left in Fig. 4). Another binding site on IL-4 that affects IL-4 signaling involves residues in the D helix, but the identity of the receptor subunit that binds to this site is unknown. It could be a second molecule of IL-4R, as in the model (receptor *A*-chain); however, the IL-2 receptor γ-chain (IL-2Rγ) has been shown to participate in at least 1 IL-4 signaling pathway, suggesting that an IL-4/IL-4R/IL-2Rγ ternary complex is formed (Kondo et al., 1993; Russell et al., 1993).

The net charge on the N-terminal IL-4R domain is $-7e$, assuming standard amino acid residue protonation states at pH 7. Accordingly, the electrostatic potential of IL-4R is mainly negative in the region of hormone–receptor interface (Fig. 4). Lys 91 is the only positively charged residue in this region. It contributes to the positive potential regions **a′** in the *B*-receptor subunit (and **c′** region in the *A*-receptor subunit).

The IL-4 has a net charge of $+7e$ (measured pI > 9; Solari et al., 1989) and is mainly surrounded by positive potential, which complements the negative potential of IL-4R at the interface (Figs. 4, 9). The only negative region **a** in the A–C face of the IL-4 bundle (residues Asp 4 and Glu 9) matches the positive region **a′** in the interface region of IL-4R. Mutation of Glu 9 to Lys leads to a 1,400-fold drop in receptor binding affinity, showing the importance of this region (Kruse et al., 1993). Interestingly, another negative region on IL-4, the region **c** (residues Glu 19, Glu 26, Asp 31, Glu 110, and Glu 114) located in the A–D face of the bundle near the expected second receptor binding site of IL-4, could interact with the positive region **c′** of the receptor *A*-chain in the putative homodimeric receptor complex. However, the hormone–receptor electrostatic potential complementarity in the region **c**-**c′** is not as precise as in the region **a**-**a′**. The negative region **c** on IL-4 is larger than the region **a** and not only complements the positive region **c′** of the receptor model but also clashes with its encircling negative potential. Considering this together with the negative region **b**, also located on the A–D face of the IL-4 bundle, complementarity of the electrostatic potentials in this part of the model is disrupted, suggesting that another subunit, different from the IL-4R, could bind to this site.

This conclusion is supported by estimates of the interaction energy between IL-4 and IL-4R (Fig. 5). In contrast to the case

[3] Binding of hGH to the receptor subunits takes place sequentially (Cunningham et al., 1991): first to regions **a′**, **b′**, **d′**, and **f′** of the receptor (binding site I) and then to regions **a′**, **c′**, and **e′** of the receptor (binding site II).

[4] Note that the receptor subunit was modeled from C_α coordinates without taking electrostatic interactions into account (see Methods). Favorable orientations may be more precisely defined for a full crystallographic coordinate set.

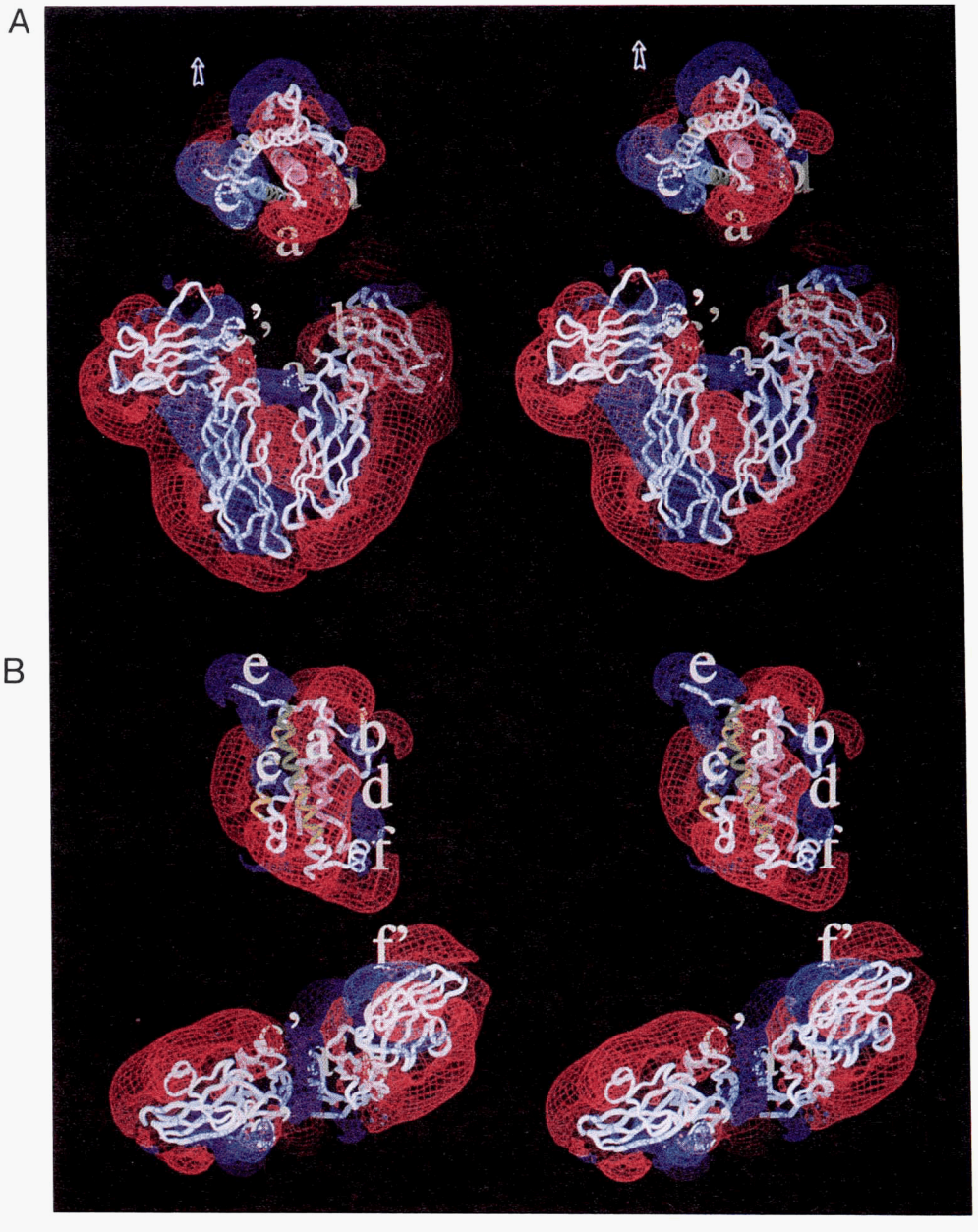

Fig. 2. Electrostatic isopotential surfaces for the hGH and the hGH receptor superimposed on ribbon diagrams of the molecules. Ribbon diagrams were created using the Insight II program. The A, C, and D helices of the hGH are colored green, yellow, and magenta, respectively. The A and D helices compose site I, which binds to the *A*-chain of the receptor. The C and A helices form site II, which binds to the *B*-chain of the receptor after the *A*-chain has bound (Cunningham et al., 1991). The potentials for the hGH molecule and the hGH receptor dimer were calculated separately using atomic coordinates from the hGH/hGH-receptor complex. The blue contours represent the isopotential surface at $+0.5kT/e$, the red contours correspond to the isopotential surface at $-0.5kT/e$, where k is Boltzmann's constant, T is absolute temperature, and e is the electronic charge. The potential at points within 2 atomic radii of protein atoms was set to 0 so that the potential surrounding the molecules could be viewed more clearly. Six regions of complementarity in the potentials are labeled by the letters **a′**–**f′** in the receptor potential and letters **a**–**f** in the hormone potential (see text for corresponding residue numbers). These regions come into contact upon formation of the ternary complex, and the letters are positioned so that complementary pairs (**a** and **a′**, **b** and **b′**, etc.) superimpose in space in the complex. **A:** Side view of the ternary complex (with the hGH translated out of the binding site) seen along the axis of the hormone binding groove of the receptor dimer in the direction parallel to the membrane surface. The groove is denoted by letter **a′**. From this view, letters **c**, **c′**, **d**, and **d′** are directly behind letters **e**, **e′**, **b**, and **b′**. **B:** Facial view of the complementary hormone–receptor surfaces of the ternary complex. The hGH molecule and the receptor dimer, taken in the orientation of Figure 2A, are rotated around a horizontal axis in the plane of the page by −90° and +90°, respectively. In this orientation, the axis of the hormone binding groove is perpendicular to the bottom of the page.

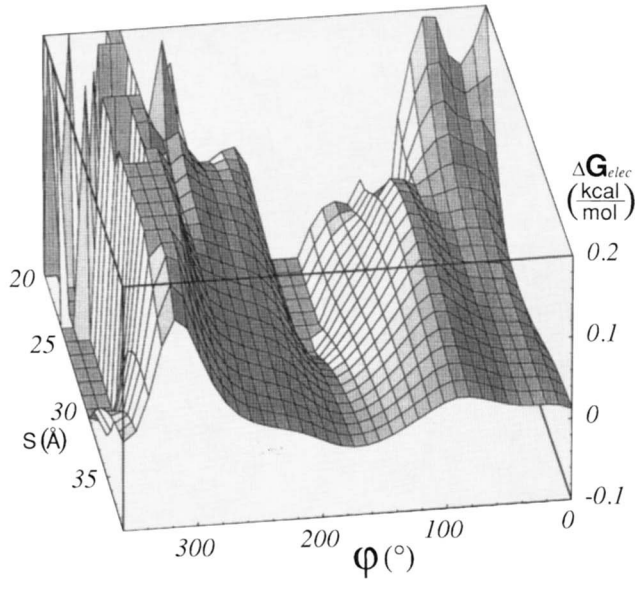

Fig. 3. Estimate of the electrostatic interaction energy of hGH and the *A*-chain of its receptor as a function of their mutual orientation. This is calculated from Equation 1 according to the test charge approximation for the receptor partial atomic charges in the electrostatic potential of the hormone. The immersion of the receptor charges in low dielectric is not taken into account. However, although this affects the magnitude of ΔG_{elec}, it has little effect on its observed dependence on angle (data not shown). The receptor molecule was translated in 1-Å intervals out of its crystallographic position in the hGH/hGH-receptor complex in the direction of the membrane surface and, at each separation, it was rotated in steps of 10° around the center of geometry of the receptor dimer in a plane parallel to the membrane surface. At small hormone–receptor separations, very high energies were obtained because of steric overlap between the molecules on rotation. Thus, the energy is displayed only in the range −0.1 to +0.2 kcal/mol and at the separation distance of 20–40 Å. The energy surface displays 2 low-energy regions – one is at about 150–180° and the other is at about 340–360° – revealing 2 orientations of the hormone–receptor system that have favorable electrostatic interactions.

for hGH and its receptor, the estimated electrostatic interaction energy for IL-4 and each of the modeled IL-4 receptor chains is favorable at only 1 orientation of the IL-4R. This corresponds to the binding site for the IL-4 receptor *B*-chain involving the A and C helices of IL-4 (left in Fig. 4). Thus, the electrostatic potential of IL-4 supports a hetero-oligomeric model for IL-4 receptor.

The IL-2Rγ is likely to be a better substrate for the receptor binding site on the A–D face of the IL-4 bundle than the IL-4R because its N-terminal domain has a smaller negative charge. According to the alignment of both sequences (Fig. 6), there are 10 acidic and 9 basic residues in the part of the IL-2Rγ sequence homologous to the N-terminal domain of IL-4R, and it may be expected that the more balanced electrostatic potential of IL-2Rγ fits the heterogeneous potential of the A–D face of the IL-4 helix bundle better.

Comparison of the electrostatic potentials of the hGH/hGH-receptor complex and the model IL-4/IL-4-receptor complex shows that, although there are differences between the electrostatic potentials of both hormones and differences between the electrostatic potentials of the receptors, for each hormone–receptor pair, the potentials complement each other in the interface region and, thus, can be used as descriptors of hormone–receptor recognition.

Structural comparison of growth factors

The considerable sequence similarity among the extracellular parts of hematopoietin receptors is thought to reflect the common ancestry not only of the receptors themselves but also of their ligands and of other components constituting the hormone–receptor system (Bazan, 1990). Indeed, despite the very low sequence similarity between the signal-transducing proteins, which normally would not allow them to be assigned to the same phylogenetic group, all 5 cytokines studied here share the same conserved 3D fold. The spatial conservation of both the cytokines and their receptors suggests that the 3D structure of their complexes may also be conserved.

Immunochemical experiments and experiments on recombinant analogs of murine (Zurawski & Zurawski, 1989, 1992; Zurawski et al., 1990) and human IL-2 (Liang et al., 1986; Weigel et al., 1989; Landgraf et al., 1992), IL-4 (Kruse et al., 1991, 1992, 1993; Ramanathan et al., 1993), and GM-CSF (Kaushansky et al., 1989; Lopez et al., 1992; Meropol et al., 1992) suggest that all of them are likely to interact with their receptors in an orientation similar to hGH (de Vos et al., 1992), i.e., with the surface involving the A, C, and D helices. Mapping of functionally important residues onto the crystal structures of IL-2 and IL-4 indicates that a region near the C-terminus is one of the potential receptor binding sites. This region corresponds to receptor binding site I of hGH in the crystal structure of the hGH/hGH-receptor complex (de Vos et al., 1992). A region similar to receptor binding site II of hGH has been identified as a potential binding site on the GM-CSF (Diederichs et al., 1991) and the IL-4[5] (Kruse et al., 1993; Ramanathan et al., 1993; Müller et al., 1994). Therefore, the 3D structures of GM-CSF, IL-2, and IL-4 were superimposed on the crystal structure of hGH by fitting helices C, A, and D of the receptor binding surface (Table 1). As can be seen in Figure 7 and Kinemage 1, the superimposed helical fragments differ more in length than in mutual orientation.

The G-CSF structures differ from those of other cytokines studied in that the 4-α-helix bundle has a more regular topology. The α-helices are longer and thus more regularly packed, but the most striking feature is that the B helix (which is short or distorted in other cytokines) is equal in length to the other 3 helices of the bundle. Thus, it is reasonable to consider an alternative location of the receptor binding site on G-CSF involving the B helix.

Experimental data concerning G-CSF do not allow an unequivocal assignment of its receptor binding surface. Mutations resulting in significantly decreased activity have been found in all 4 helices of hG-CSF (Kuga et al., 1989; Layton et al., 1991; Ishikawa et al., 1992). Thus, 2 possible superpositions of hG-CSF (Hill et al., 1993) and bG-CSF (Lovejoy et al., 1993) were considered. In one, the orientation of G-CSF is similar to that of the other 3 cytokines. In the other, the G-CSF is rotated 90° around the axis of the helical bundle and 180° around an axis

[5] Note that the sequence of IL-4 receptor subunit binding is opposite to that of hGH, i.e., the A and D helices of hGH are part of binding site I, whereas in IL-4, the same helices constitute binding site II (Kruse et al., 1993).

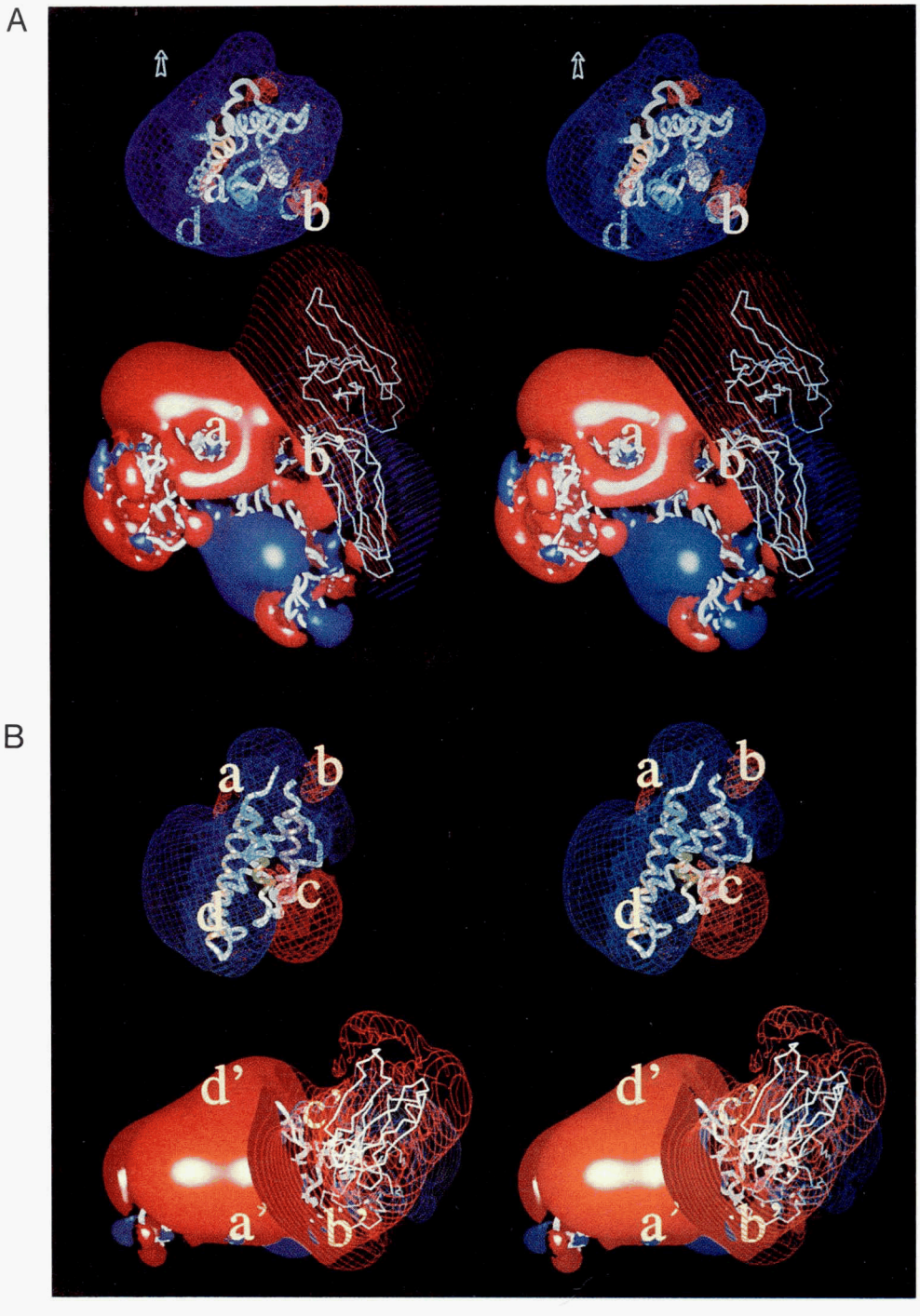

Fig. 4. Electrostatic isopotential surfaces for the IL-4 NMR structure and a model of its receptor composed of 2 identical IL-4R subunits superimposed on ribbon diagrams and the backbone of the molecules. The A, C, and D helices of the IL-4 are colored green, yellow, and magenta, respectively. The C and A helices form site I, which binds the IL-4R (Kruse et al., 1993). This part of the receptor (the *B*-chain in the model) is shown by a ribbon with solid surface presentation of the electrostatic potential. The *A*-chain of the modeled homodimeric receptor and the corresponding part of the potential contour are shown in line presentation because experimental evidence suggests that a hetero-oligomeric receptor complex is formed with a different receptor subunit instead of the *A*-chain (see text for details). The electrostatic potentials for IL-4 and for the IL-4 receptor dimer were calculated and are displayed as described in the caption to Figure 2. Three regions of negative potential and 1 region of positive potential in the hormone–receptor interface of IL-4 are labeled by the letters **a–c** and **d**, respectively. Counterpart regions in the IL-4-receptor model are labeled by the letters **a′–d′**. The **(A)** side and **(B)** facial views of the model given are equivalent to those for hGH in Figure 2.

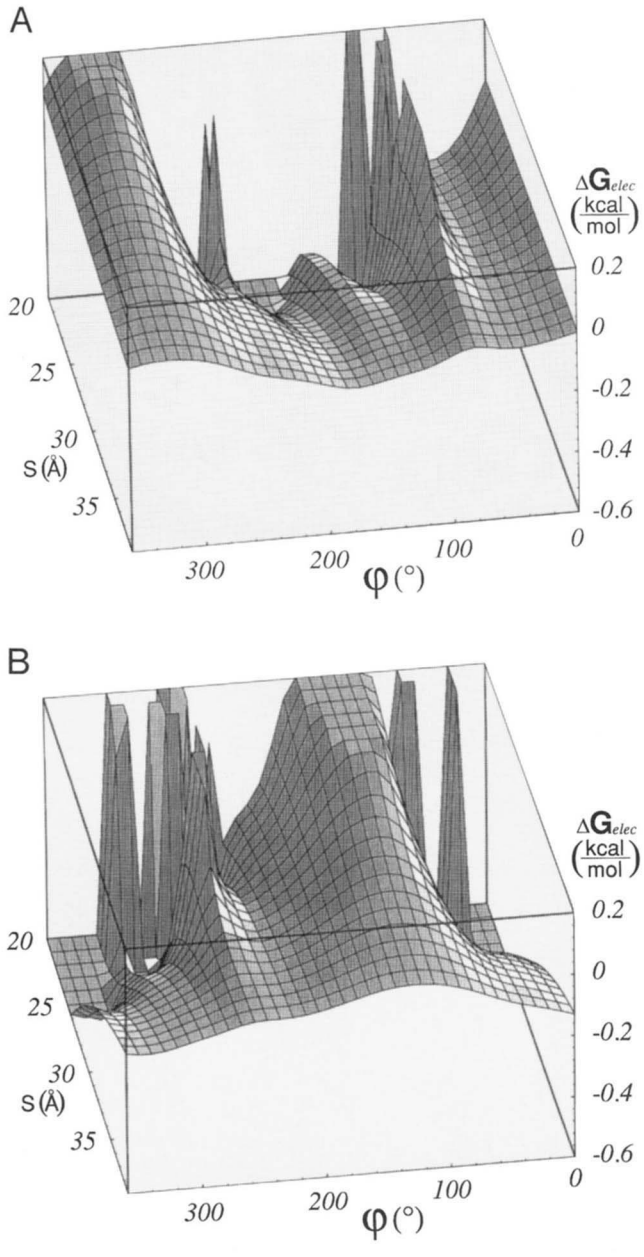

Fig. 5. Estimate of the electrostatic interaction energy between the IL-4 NMR structure and the modeled **(A)** IL-4R *A*- and **(B)** *B*-chains. Calculations were done as described in the legend to Figure 3. Both energy surfaces display only 1 low-energy region occurring at about 0° for the *B*-chain and 180° for the *A*-chain. The region of favorable hormone–receptor electrostatic interactions corresponds to the orientation of the receptor *B*-chain in the ternary model.

perpendicular to the bundle axis, resulting in a superposition that brings the B, C, and A helices of G-CSF into correspondence with the C, A, and D helices of hGH ("alternative" superposition in Table 1).

Symmetry of electrostatic potential

Human growth factor binds 2 identical receptor molecules sequentially (Cunningham et al., 1991). In the complex, the positions of the 2 receptor molecules are related by an approximately 2-fold symmetry axis. One molecule can be superimposed on another with an RMSD in C_α atoms of 1.0 Å by a rotation through 159° and by a translation of 8 Å (de Vos et al., 1992). As a consequence, the electrostatic potential in the interface region of the receptor dimer has 2-fold rotational symmetry around an axis perpendicular to the cell membrane surface through the center of the hormone binding groove of the hGH ternary complex (region **a′** in Fig. 2B). Twofold rotational symmetry should be a general property of homodimeric receptor complexes consisting of 2 Ig-like receptor molecules (Fig. 8). Heterosubunit receptor complexes will not necessarily possess such symmetry.

Although an Ig-like receptor consisting of 2 identical subunits would be expected to possess 2-fold rotational symmetry, a hormone that acts as a single-domain monomer is not required to have such symmetry. Both subunits of the hGH receptor use essentially the same residues to interact with the hGH, but there is no obvious structural similarity between the 2 binding sites on hGH. Nevertheless, the electrostatic potential of hGH has 2-fold rotational symmetry about the same axis as the receptor dimer and is complementary to the electrostatic potential of the receptor (Figs. 2, 9A), i.e., although the molecular surfaces are different at the 2 receptor-binding sites on the hormone, the charge distributions are similar. Four regions of positive potential (regions **b**–**e**) surrounding the negative center of symmetry of the receptor-binding interface (region **a**) form a roughly symmetrical potential in which region **b** is positioned approximately symmetrically to region **c**, and region **d** is positioned approximately symmetrically to region **e**.[6]

The symmetry of the hGH electrostatic potential can also be discerned from the bimodality of the interaction energy surface given in Figure 3. The interaction energy for the receptor chain is most favorable at 2 angle ranges that are approximately 2-fold

[6] Note that the symmetry is approximate because the complementary receptor subunits are superimposable by rotation through an angle less than 180° (159°).

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IL4R    vlqEptcvsD ymsistcEwK mngptncstE lRllyqlvfl -lsEahtcip
IL2g    plpEvqcfvf nvEymnctwn sssEpq-ptn ltlhywyKns DnDKvqKcsh

IL4R    -Ennggagcv chllmDDvvs aDnytlDlwa gqqllwKgs- -fKpsEhvKp
IL2g    ylfsEEitsg cqlqKKEihl yqtfvvqlqD pREpRRqatq mlKlqnlvip
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Fig. 6. Sequence alignment of the N-terminal domains of human IL-4R and human IL-2Rγ. Capital letters denote charged residues, and positively charged residues are shown in italics.

Table 1. *Residues constituting structurally conserved regions of the hGH and the cytokines*

Molecule	Superimposed residues			RMSD[a] (Å)
hGH	Pro 5–Gln 29	Tyr 111–Glu 119	Cys 165–Ser 184	2.0
IL-2	Ser 6–Asn 30	Ile 86–Leu 94	Thr 113–Leu 132	
hGH	Ser 7–Ala 24	Ser 106–Gly 126	Lys 168–Ser 184	1.1
IL-4	Asp 4–Lys 21	Arg 75–Gly 95	Asn 111–Cys 127	
hGH	Pro 5–Leu 20	Leu 113–Glu 129	Cys 165–Val 180	1.6
GM-CSF	Glu 14–Ser 29	Ser 69–Lys 85	Thr 102–Ile 117	
hGH	Ser 7–Glu 32	Asn 109–Thr 123	Asn 159–Lys 172	1.7
hG-CSF	Gln 11–Cys 36	Thr 105–Gln 119	Arg 147–Phe 160	
hGH	Ala 13–Glu 30	Ser 108–Asp 116	Leu 157–Phe 166	2.13[b]
hG-CSF	Leu 103–Gln 119	Arg 22–Ala 30	Gly 73–Leu 82	
hGH	Arg 8–His 21	Ser 106–Leu 128	Cys 165–Gln 181	1.8
bG-CSF	Ser 13–Gln 26	Thr 103–Leu 125	Gln 157–Ala 173	
hGH	His 18–Ala 24	Asp 107–Met 125	Asn 159–Asp 169	1.7[b]
bG-CSF	Leu 109–Ala 115	Cys 18–Leu 36	Leu 76–Tyr 86	

[a] RMSD for C_α atoms of listed residues.
[b] Alternative superposition.

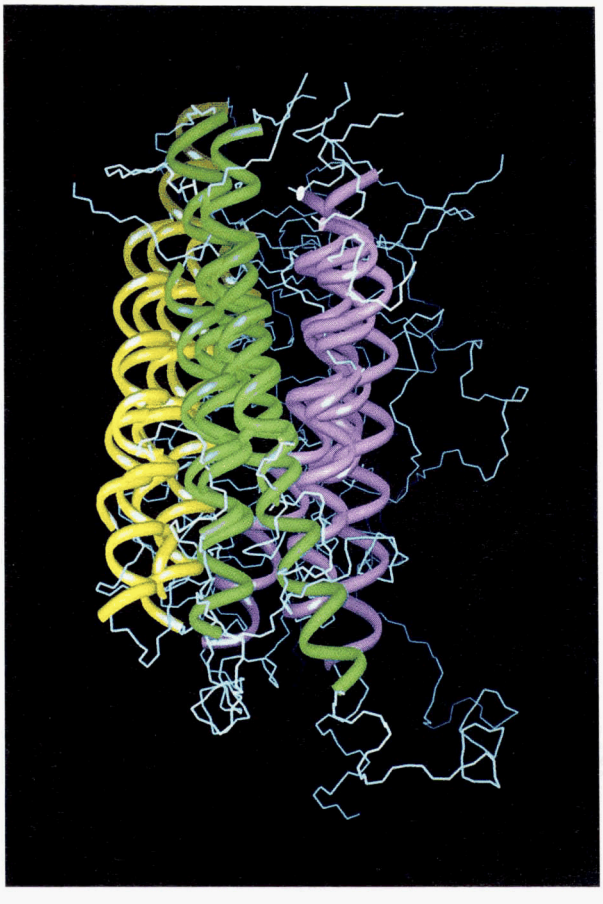

Fig. 7. Best-fit superposition of the C_α atoms from the A (green), C (yellow), and D (magenta) helical segments of GM-CSF, G-CSF, IL-2, and IL-4 onto those of hGH. Residues are equivalenced as shown in Table 1.

symmetric and correspond to the regions deduced from graphical analysis.

A direct measure of the symmetry of the electrostatic potential of the hormone was derived by calculating an electrostatic potential self-similarity index as defined by Equation 2. This de-

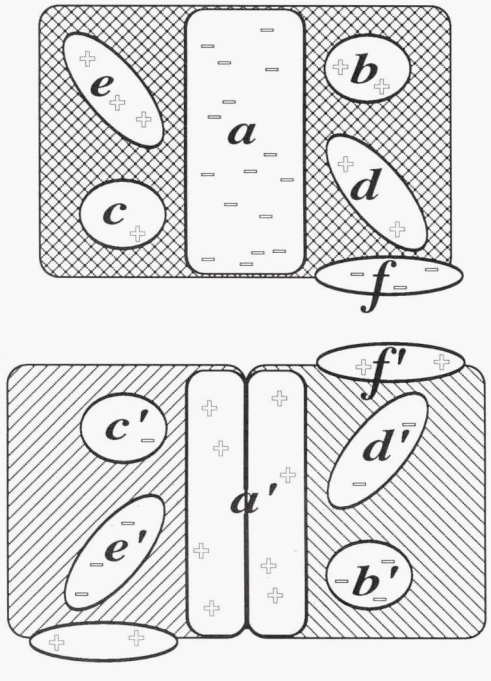

Fig. 8. Schematic diagram of the electrostatic potential surfaces (upper, hormone; lower, receptor) given in Figure 2B. A homodimeric receptor consisting of 2 identical subunits has 2-fold rotational symmetry about the axis marked with letter *a'*.

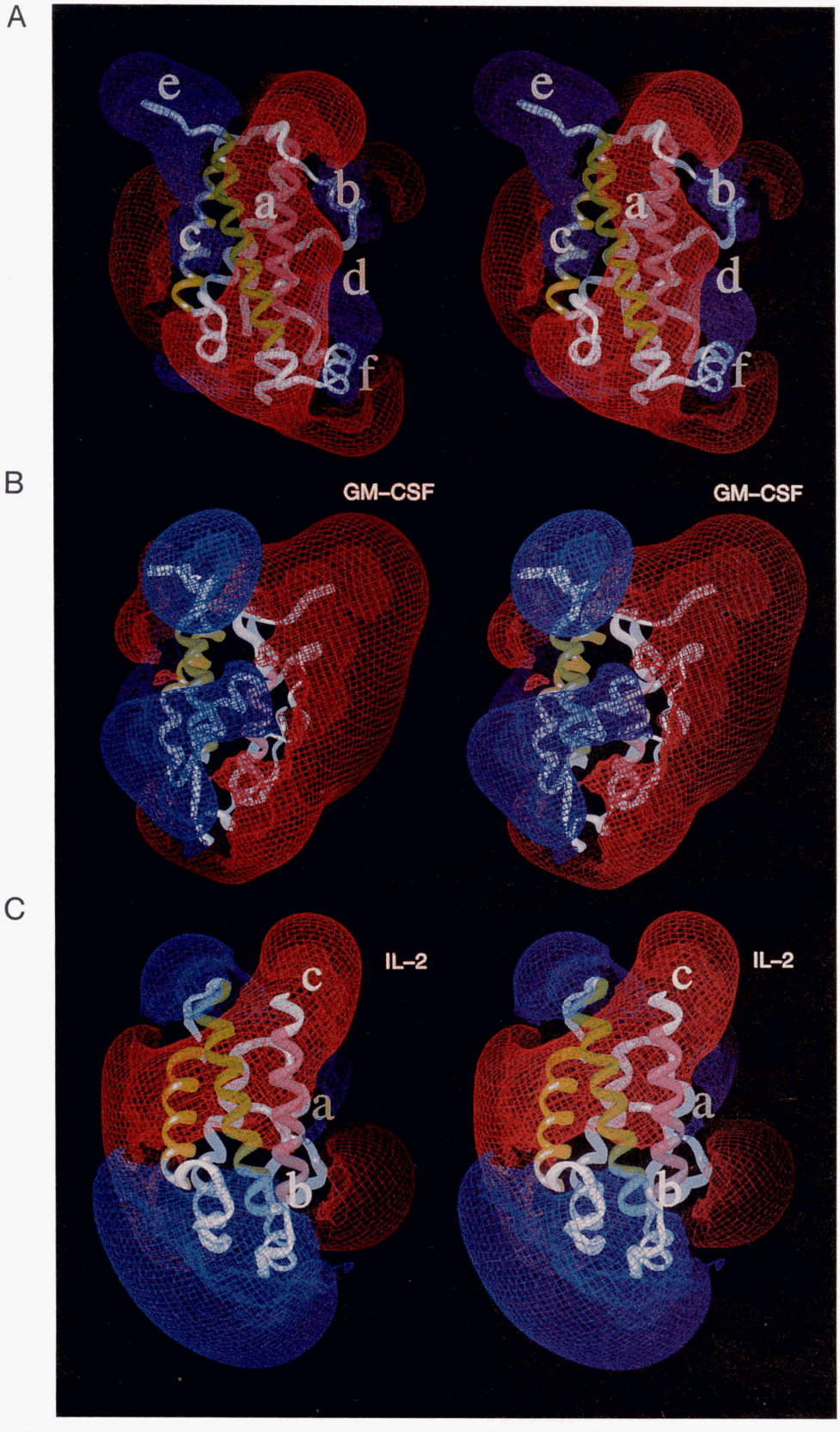

Fig. 9. Electrostatic isopotential surfaces of hGH, GM-CSF, IL-2, IL-4, and G-CSF in solution superimposed on ribbon diagrams of the molecules. Potential contour levels, colors, and molecule presentation are as in Figure 2. Molecules are viewed in the same orientation as hGH in Figure 2B. **A:** hGH. The positive regions **b** and **c** are located approximately symmetrically around the central negative region **a**, as are regions **d** and **e**. **B:** GM-CSF. Electrostatic potential lacks 2-fold rotational symmetry on the surface formed by the C–A–D helices. (*Continues on facing page.*)

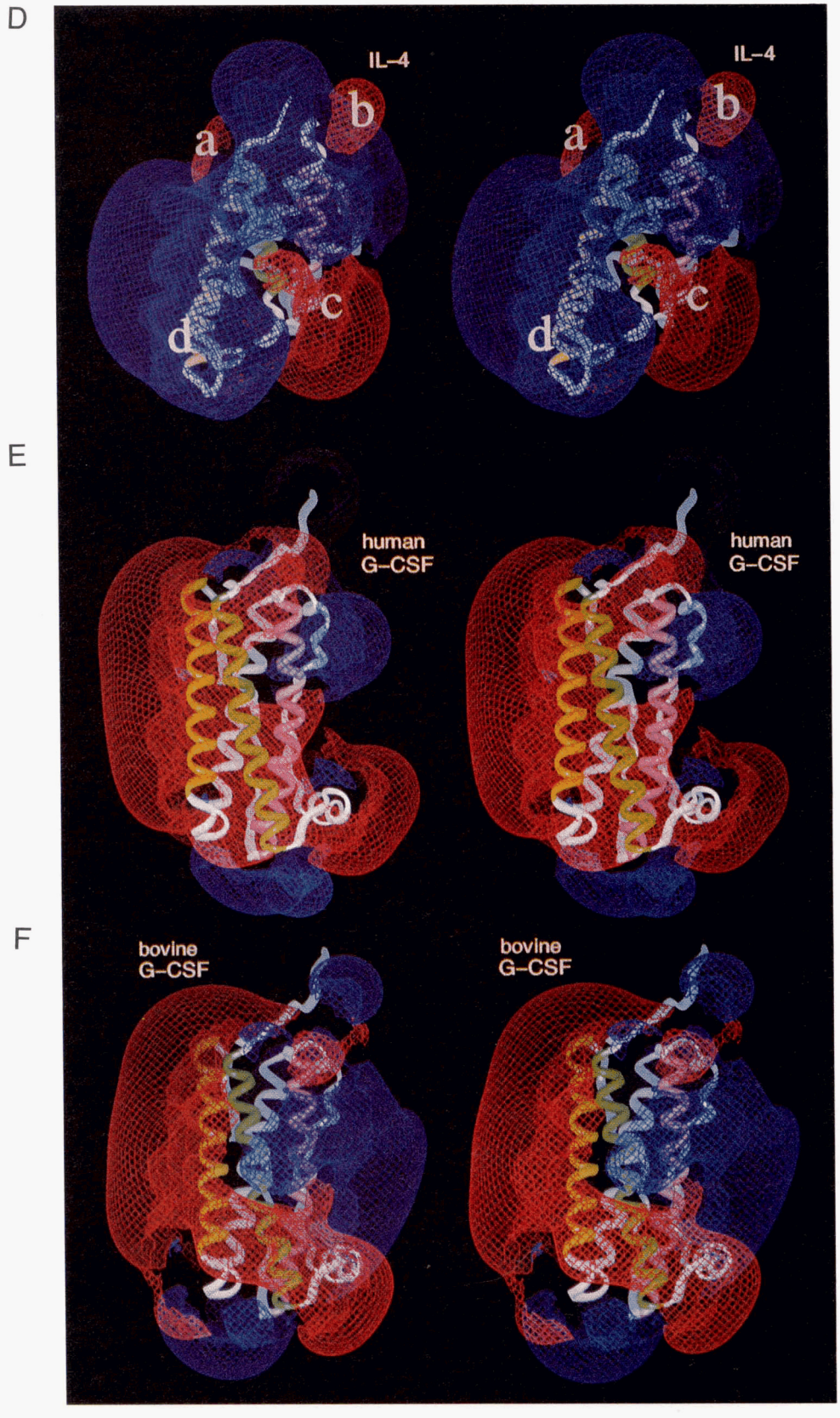

Fig. 9. *Continued.* **C** (*facing page*): IL-2. The region of low potential between points **a** and **b** and the region of negative potential denoted by letter **c** disrupt the 2-fold rotational symmetry of the potential. **D:** IL-4 (NMR structure; Müller et al., 1994). Three regions of negative potential in the interface region are denoted by letters **a**, **b**, and **c**. Letter **d** shows a positive region that breaks 2-fold rotational symmetry. **E:** hG-CSF. See text for details. **F:** bG-CSF. See text for details.

scribes the similarity of the electrostatic potential at different orientations. The self-similarity indexes of hGH characterize its potential around the receptor binding face of the bundle as 2-fold symmetrical (Fig. 10A). Such a symmetric electrostatic potential, resulting from a nonrandom distribution of charged residues on the receptor-binding surface of the hormone, is likely to be a necessary condition for specific complexation with a homodimeric receptor.

In contrast to the hGH (Fig. 9A), the electrostatic potentials surrounding the proposed receptor binding surfaces of both GM-CSF (Fig. 9B) and IL-2 (Fig. 9C) lack 2-fold rotational symmetry. The electrostatic potential of GM-CSF is mainly positive around the C helix and negative around the D helix. The asymmetry of the potential of IL-2 is largely due to a patch of apolar residues at the C-terminal end of the A helix and along the D helix (denoted by the region **a–b** in Fig. 9B; and comprising residues Leu 25, Ile 28, Ala 50, Thr 51, Leu 53, Leu 70, Val 115, Thr 123, and Phe 124) and a region of negative potential around the C-terminus (region **c** in Fig. 9C). There are no equivalent regions in the electrostatic potential of IL-2 on the opposite side of the presumed 2-fold symmetry axis. The electrostatic potential similarity indexes of GM-CSF and IL-2 given in Figure 10B and C confirm the graphical analysis showing that the electrostatic potentials in the interface region of both hormones are most dissimilar at opposite sides of the interface region (180° apart). Unlike hGH, both GM-CSF and IL-2 are thought to form complexes with hetero-oligomeric receptors composed of at least 2 different noncovalently bound polypeptide chains (Miyajima et al., 1992; Kastelein & Shanafelt, 1993; Minami et al., 1993). The electrostatic potential distributions of GM-CSF and IL-2 are consistent with these experimentally based models.

Electrostatic potentials calculated for both the crystal (Wlodawer et al., 1992) and the NMR (Müller et al., 1994) structures of human IL-4 are very similar (not shown). The configuration of the IL-4 electrostatic potential in the interface region of the hormone was discussed earlier in connection with the IL-

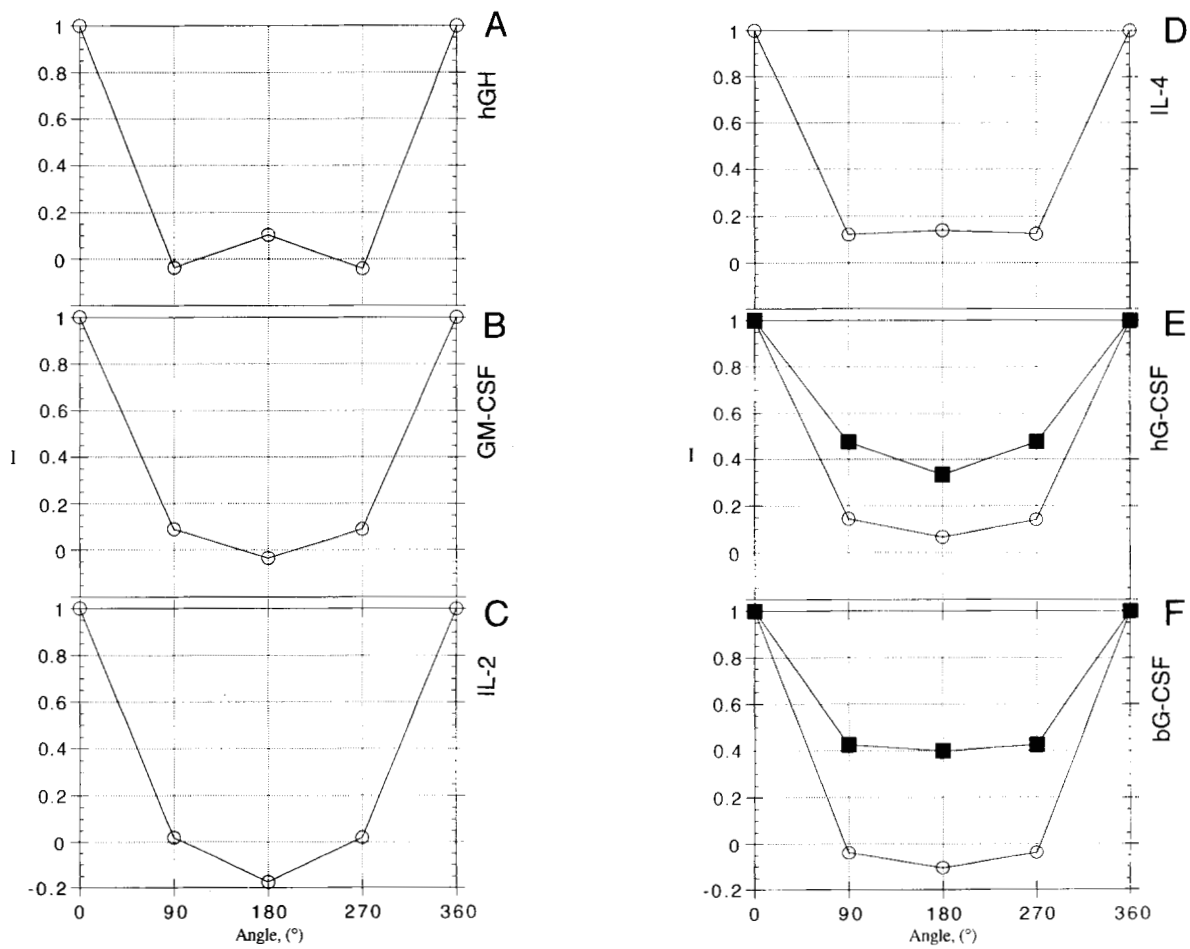

Fig. 10. Indexes of electrostatic potential self-similarity around the receptor binding faces of (**A**) hGH, (**B**) GM-CSF, (**C**) IL-2, (**D**) IL-4, (**E**) hG-CSF, and (**F**) bG-CSF. For hGH, the electrostatic potential grids calculated for the orientation of Figure 2B were rotated by 90°, 180°, and 270° around the axis perpendicular to the page. For each angle, the self-similarity of the electrostatic potential over the hormone–receptor interface region was calculated from Equation 2. The higher the index value, the more similar the electrostatic potentials. Calculations were repeated for each of the other hormones superimposed on hGH with C_α atoms as listed in Table 1. Black squares in E and F represent indexes for the "alternative" binding surface of G-CSF with helices B–C–A at the interface.

4R model. Three distinct regions of negative potential are present on the C-A-D helix surface of the hormone (denoted by letters **a**, **b**, and **c** in Fig. 9D). Together they define an asymmetric electrostatic potential pattern: 2 of these 3 negative regions, **a** and **c**, are positioned symmetrically with respect to the presumed 2-fold symmetry axis, but the third, region **b**, has no counterpart negative potential on the other side of the symmetry axis (region **d**). This suggests that the binding site on the A-D face of the IL-4 helix bundle is likely to interact with a receptor subunit different from IL-4R, which binds to the C-A face of the hormone.

Two crystal structures of G-CSF, human (Hill et al., 1993) and bovine (Lovejoy et al., 1993), were available. Despite having different net charges ($-4e$ on hG-CSF [experimental pI = 5.9–6.1; Clogston et al., 1992] and $-1e$ on bG-CSF), the electrostatic potential distributions are similar (Fig. 9E,F). Differences at 8 charged residues in these 2 proteins have a fairly limited effect on the overall electrostatic potential around the hormones. Thus, similarity is consistent with the cross-reactivity observed in vivo between these 2 species of G-CSF (Cullor et al., 1990).

Analysis of electrostatic potential similarity indexes shows that the electrostatic potential around the C-A-D surface of the bundle lacks 2-fold symmetry (Fig. 10E,F). For both hG-CSF and bG-CSF, 2-fold symmetry is more apparent for the B-C-A surface, which was discussed as an alternative putative receptor binding surface earlier. High index numbers for all the calculated angles for this surface indicate the relative homogeneity of the electrostatic potential over the surface, making it a possible binding surface for a homodimeric receptor. Indeed, when viewed in the same orientation as the 4 other cytokines studied, the electrostatic potentials of G-CSF lack the 2-fold rotational symmetry expected to be necessary for the formation of homodimeric high-affinity hormone-receptor complexes (Fig. 9E,F). The C helix is surrounded by a large region of negative potential stretching over the end of the A helix to the D helix and covering the N-terminal part of it. The rest of this surface is covered by positive potential. From this point of view, the electrostatic potential indicates that G-CSF is likely to be a ligand with higher affinity to a hetero-oligomeric than a homodimeric receptor. On the other hand, the electrostatic potential over the "alternative" B-C-A surface of G-CSF (left side of G-CSF in Fig. 9E,F) is negative and, thus, could bind to a positively charged region on a homodimeric receptor.

Analysis of the electrostatic potentials of hGH, GM-CSF, IL-2, and IL-4 indicates that the distribution of the electrostatic potential around the binding surface of the hormones correlates with the known modes of receptor binding. It indicates whether it is possible for a hormone to induce formation of a high-affinity homodimeric receptor complex rather than a hetero-oligomeric receptor complex.

Discussion

The complementarity of the hormone and receptor electrostatic potentials of hGH and IL-4 suggests that not only hydrophobic (de Vos et al., 1992) but also electrostatic interactions are important for the formation of a signal-transducing complex. In the absence of complete structural data, it is not possible to compare the actual contributions of different types of interactions to the binding energy of the complexes formed. Thus, we have examined the electrostatic potentials of the hormones and their receptors, and also the hormone-receptor interactions under the test-charge approximation. These quantities are rather robust to possible errors in the modeling of atomic coordinates, in contrast to the steric, hydrogen bond, and desolvation components of hormone-receptor interactions for which high-quality structures are likely to be necessary. We have analyzed the electrostatic properties of unbound hormones and their receptors.[7] Electrostatic interactions in the hormone-receptor complexes may differ from those for the unbound molecules because new ion pairs may form in the complex, conformational changes may occur, and because the dielectric boundary is different. However, electrostatic interactions affect not only binding energy but also the rate at which hormone-receptor complexes are formed. Diffusion of the hormone up to the receptor is guided by long-range electrostatic interactions (described by the electrostatic potentials of the unbound hormone and receptor). Recent measurements of hGH binding kinetics revealed the importance of charged residues in determining the kinetics of binding (Cunningham & Wells, 1993). Mutations of Arg 64, Glu 65, Arg 167, Glu 174, and Arg 178 to alanine reduce the hGH binding rate more sharply than others suggesting "that electrostatic interactions are the most important side-chain determinants in guiding the hormone to the receptor" (Cunningham & Wells, 1993). Interaction energy estimates (Fig. 3) suggest that the hormone may be rotationally steered to dock correctly to the receptor by electrostatic interactions at considerable distances from the receptor.

Electrostatic interactions may also play a role in determining the order of formation of hormone-receptor complexes. On activation, the hGH receptor assembles in 2 stages (Cunningham et al., 1991): (1) binding of the first receptor molecule (*A*-chain) to site I of the hGH (Fig. 2) and (2) binding of the second receptor molecule (*B*-chain) to site II on hGH and dimerization of the receptor. The fifth peripheral α-helix of hGH, whose residues contribute to regions **d** and **f** in the electrostatic potential (Fig. 2), is probably one of the determinants of the sequential character of hormone-receptor binding. It is located near binding site I of the hormone and disrupts the symmetry of the electrostatic potential. The binding of this peripheral helix is also the main reason for the difference in the surface areas of the receptor subunits buried at the hormone-receptor interface (1,300 Å^2 for the *A* chain, 900 Å^2 for the B-chain; de Vos et al., 1992). The additional interactions of this part of the hormone probably facilitate binding of the first receptor molecule during the first stage of hormone-receptor complex formation. The second stage of complexation is probably promoted by the stretch of negative potential along the A helix of hGH (region **a**). By interacting with the residues of the positive region **a'** of the receptor *A*-chain in the first stage of hormone-receptor complex formation, it changes the electrostatic potential around the *A*-chain of the receptor (not shown) and thereby reduces electrostatic repulsion between the 2 identical receptor subunits.

Similar events during formation of the functional receptor are expected for IL-4, although the order of receptor subunit bind-

[7] All hormone electrostatic potentials were calculated for unbound hormones except for hGH, for which the potential was calculated for the hormone taken from the ternary hormone-receptor complex. Electrostatic potentials calculated for receptor monomers from the hormone-receptor complexes analyzed (not shown) are similar to those of their dimers over the hormone binding surfaces.

ing to sites on the A–C and the A–D faces of the IL-4 bundle is the reverse of that of hGH and its receptor (Kruse et al., 1993). The importance of charged residues in determining the sequential binding is suggested by experiments with recombinant analogs of human IL-4 (Kruse et al., 1993; Ramanathan et al., 1993). One of the major differences distinguishing IL-4 from other cytokines is a long patch of positive residues composed of Arg 75, Arg 77, Arg 81, Lys 84, Arg 85, and Arg 88 in the C helix (Wlodawer et al., 1993). A single point mutation of Arg 85 to aspartic acid leads to a 50% reduction in hormone activity (Kruse et al., 1993; Ramanathan et al., 1993), and substitutions of Lys 84 (Ramanathan et al., 1993) and Arg 88 (Kruse et al., 1993; Ramanathan et al., 1993) by aspartic acid result in nearly complete loss of binding activity, indicating that the basic character of this region is vital. On the other hand, single point mutations of residues Ser 125, Cys 127, and Ser 128 to aspartate in the negative region **b**, which is symmetric to region **d**, do not significantly influence the binding properties of IL-4 (Kruse et al., 1993) even though this region is involved in receptor binding (Kruse et al., 1991, 1993; Ramanathan et al., 1993). This indicates that these 2 regions of IL-4 interact with the receptor differently, providing further evidence for a heterodimeric receptor complex. Site-directed mutagenesis of these regions, e.g., the introduction of positively charged residues in region **b** near the C-terminus, could provide additional valuable information about the receptor binding properties of IL-4.

Although several studies postulate that IL-4 binds to a homodimeric receptor (Bamborough et al., 1993; Wlodawer et al., 1993), experimental evidence supports a hetero-oligomeric model for the functional IL-4 receptor: (1) IL-4 induced oligomerization of the 130-kDa high-affinity IL-4R assumed in the model of Bamborough et al. (1993) has not been detected in Scatchard analysis for either the complete molecule or its extracellular part (Kruse et al., 1993); and (2) recently, several groups have detected IL-4 interaction with other transmembrane molecule(s): a low-affinity IL-4 receptor with a molecular weight of 65–75 kDa has been identified (Fanslow et al., 1993) and the 64-kDa IL-2Rγ has been shown to affect IL-4 signal transduction and to promote IL-4/IL-4R complexation (Kondo et al., 1993; Russell et al., 1993).

From the present analysis, it is not possible to distinguish reliably the actual binding mode of G-CSF from the 2 binding modes discussed. However, several pieces of experimental evidence favor the suggestion of a different binding surface for the G-CSF receptor on the G-CSF bundle compared to the other hormones considered. The importance of the D helix of G-CSF for receptor binding may be questioned because the mature hormone, deprived of 53 C-terminal residues constituting the D helix and the C–D interhelix loop, has been found to retain activity, albeit significantly reduced, and to have no detectable antagonistic activity (Layton et al., 1991). In contrast to IL-4 (Kruse et al., 1993), hGH (Cunningham & Wells, 1989), and GM-CSF (Altmann et al., 1991), single point mutations in the B helix of hG-CSF have produced mutants with no detectable activity (Kuga et al., 1989), suggesting that the B helix of G-CSF may participate in receptor complex formation.

A homodimeric model for the G-CSF receptor has been introduced by Fukunaga and coworkers based on biochemical data (Fukunaga et al., 1991). They have identified 1 ligand binding component of the G-CSF receptor (Fukunaga et al., 1990a, 1990b) and observed bilinear curves in Scatchard analysis indicating G-CSF-induced oligomerization of this receptor binding component (Fukunaga et al., 1990a). Signal transduction experiments with chimeric molecules of hGH and murine G-CSF may also lend support to a homodimeric model of the functional G-CSF receptor (Ishizaka-Ikeda et al., 1993). However, signal transduction after transformation of G-CSF-independent cells with the G-CSF gene has been detected in IL-3-dependent cell lines and not in an IL-2-dependent line (Fukunaga et al., 1991). This suggests the possibility that a receptor subunit may be shared with IL-3, which is already known to have a receptor subunit in common with the GM-CSF and IL-5 receptors (Sakamaki et al., 1992; Goodall et al., 1993).

The electrostatic potentials suggest that a search for an unusual organization of the G-CSF hormone–receptor complex may be productive. Further experiments with mutants of G-CSF with altered electrostatic properties could help to clarify the stoichiometry of its hormone–receptor complex and to identify its binding surface.

Advances in recombinant DNA technology and the use of monoclonal antibodies have facilitated the progress of functional studies of cytokines and growth factors. However, the detailed mechanism of hormone–receptor recognition is still unclear. The present study demonstrates that the electrostatic analysis of growth factors based only on the known or predicted 3D structure of the protein can suggest a general scheme for hormone–receptor interactions and thus provide useful guidelines for experimental research.

Materials and methods

Materials

The 3D structures of 6 hormones and 2 receptor complexes were used. A full coordinate set for the hGH and C_α coordinates for the hormone–receptor complex (de Vos et al., 1992) were provided by Dr. A. de Vos; structures of hG-CSF (Hill et al., 1993) and bG-CSF (Lovejoy et al., 1993) were provided by Dr. D. Eisenberg; the model of the IL-4/IL-4-receptor complex (Bamborough et al., 1993) was provided by Dr. G. Richards. The human IL-4 structure solved by NMR has been described by us (Müller et al., 1994). The crystallographic structures of human GM-CSF (Diederichs et al., 1991), human IL-2 (McKay, 1992), and human IL-4 (Wlodawer et al., 1992) used were prerelease entries of the Brookhaven Protein Data Bank (Bernstein et al., 1977).

All the calculations were carried out on a Silicon Graphics 4D/480VGX computer.

Modeling and analysis of the 3-dimensional structures

Side-chain atoms were added to the hGH receptor complex coordinate set, which consisted of C_α atoms only, according to the simulated annealing protocol of Nilges and Brünger (1991) using the X-PLOR program (Brünger, 1990). The backbone and side-chain atoms were placed at the positions of the C_α atoms. This set of coordinates was passed through a simulated annealing procedure in which the positions of the C_α atoms were always kept fixed. This consisted of 100 ps of molecular dynamics at 1,200 K followed by gradual cooling to 300 K in 6 ps and subsequent energy refinement with a modified CHARMM force field in which the electrostatic term was omitted and the

Lennard-Jones term was replaced by a softer repulsion term. This procedure was repeated 10 times to yield 10 different coordinate sets. The average structure was computed and then minimized using the full force field except for the electrostatic term.

Missing residues and side chains were added according to the sequences in the SwissProt or GenBank databases using the SYBYL molecular modeling software, version 6.0 (Tripos Associates, San Diego, California). SYBYL was also used to assign polar hydrogen atom positions. These were optimized with the AMBER united atom force field by energy minimization with all non-hydrogen atoms fixed. The protonation state of histidine residues was assigned by geometric analysis of potential hydrogen bonds. Other titratable residues were assigned their usual protonation state at pH 7. The formation of disulfide bonds was determined from experimental data and local geometries. N- and C-termini were assumed to be ionized.

Searches for 3D similarity and the superposition of 3D structures was done using the WHAT IF program (Vriend, 1990; Vriend & Sander, 1991). Each of the complete IL-2, IL-4, hG-CSF, bG-CSF, and GM-CSF α-carbon sets was searched for the best superposition on the C_α atoms of hGH. The initial superposition search was carried out using "relaxed" structure-fitting parameters (the length for the shortest segments compared, minlen = 20; the maximal error for deviation of 2 equivalenced α-carbon atoms, maxerr = 7 Å; the maximal RMSD between the compared fragments, rmserr = 3.5 Å). The final superposition of 3D structures was done under "strict" conditions (minlen = 10, maxerr = 2.5 Å, rmserr = 3.1 Å).

Electrostatics calculations

The electrostatic potentials were calculated by numerically solving the finite difference linearized PB equation using an incomplete Cholesky preconditioned conjugate gradient method as implemented in the University of Houston Brownian Dynamics (UHBD) program, version 4.0 (Davis et al., 1990).

The OPLS parameter set (Jorgensen & Tirado-Rives, 1988), with the radii of hydrogen atoms set to 1.2 Å, was used to assign atomic radii and partial charges. Dielectric constants of 78 and 2 were assigned to the solvent and the solute, respectively. The solvent-solute dielectric boundary was determined from the protein 3D structure by the method of Shrake and Rupley (1973) using a 1.4-Å radius rolling probe. Dielectric boundary smoothing (Davis & McCammon, 1991) was implemented. The ionic strength of the solvent was assumed to be 145 mM and to follow a Boltzmann distribution at 300 K. A 2-Å ion exclusion layer was used. Electrostatic boundary conditions were set using the single Debye-Hückel sphere approximation for the hormones and the multiple Debye-Hückel sphere approximation for the receptor (Davis et al., 1990). A 100^3 grid with a 1-Å spacing was used for the hormone calculations and a 140^3 grid of the same spacing was used for the receptor calculations. In order to ease the interpretation of the calculated electrostatic potential maps, the potential at grid points within the exclusion volume contained by spheres surrounding the protein atoms and having radii equal to 2 atomic radii was set to 0 before contouring and displaying the electrostatic potentials.

The electrostatic interaction energy between a hormone and its receptor was estimated as a function of their mutual orientation (rotation ψ and translation s) according to the test charge approximation for the receptor partial atomic charges q_i in the electrostatic potential $\phi_i(\psi, s)$ of the hormone:

$$\Delta G_{elec}(\psi, s) = \sum_{\forall i_{receptor}} q_i \phi_i(\psi, s), \quad (1)$$

where the sum is over all atoms in the receptor.

The symmetry of the electrostatic potential of each hormone was estimated by calculating an electrostatic potential similarity index (Carbo & Domingo, 1987). The electrostatic potentials of the hormone in initial and rotated orientations were calculated and the normalized average of their grid point product was evaluated as

$$I = \frac{\sum_i \phi_{0i}\phi_{\psi i}}{\left(\sum_i \phi_{0i}^2\right)^{1/2}\left(\sum_i \phi_{\psi i}^2\right)^{1/2}}, \quad (2)$$

where I is an index of similarity, ϕ_{0i} is the potential at point i of the initial grid, $\phi_{\psi i}$ is the potential at the same point in space of the rotated grid ($\psi = 90°, 180°, 270°$). The product grid has positive values at points where the initial and rotated potentials have the same sign and negative values where the potentials are of opposite sign. This quantity was calculated over the hormone-receptor interface region for grid points further than 2 atomic radii from all atom centers. The "interface" region was defined as the part of the grid extending from the center of geometry of the hormone in the direction of the membrane surface.

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