Hematopoietic cytokines: Similarities and differences in the structures, with implications for receptor binding

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Abstract

Crystal and NMR structures of helical cytokines—interleukin-4 (IL-4), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2)—have been compared. Root mean square deviations in the C_{α} coordinates for the conserved regions of the helices were 1–2 Å between different cytokines, about twice the differences observed for independently determined crystal and solution structures of IL-4. Considerable similarity in amino acid sequence in the areas expected to interact with the receptors was detected, and the available mutagenesis data for these cytokines were correlated with structure conservation. Models of cytokine–receptor interactions were postulated for IL-4 based on its structure as well as on the published structure of human growth hormone interacting with its receptors (de Vos, A.M., Ultsch, M., & Kossiakoff, A.A., 1992, *Science* 255, 306–312). Patches of positively charged residues on the surfaces of helices C and D of IL-4 may be responsible for the interactions with the negatively charged residues found in the complementary parts of the IL-4 receptors.

Keywords: growth factors; interleukins; receptor binding; sequence alignment; structure alignment

Many cytokines (proteins that influence cell growth and differentiation) have been identified so far. These molecules accomplish their biological role by binding to specific receptors, which in turn generate intracellular signals, initiating developmental processes in the cells (Nicola, 1989). In particular, hierarchical assembly of hematopoietic cells is regulated by a number of cytokines, which bind to distinct but related receptors. These hematopoietins include, among others, interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, and IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, and growth hormones (Boulay & Paul, 1992). Medium- to high-resolution structures of several of the hematopoietins have now been determined by X-ray crystallography and NMR. These include GM-CSF (Diederichs et al., 1991; Walter et al., 1992a), macrophage colony-stimulating factor (Pandit et al., 1992), granulocyte colony-stimulating factor (Hill et al., 1993), IL-5 (Milburn et al., 1993), the recently corrected structure of IL-2 (Brandhuber et al., 1987; McKay, 1992), and the crystal and NMR structures of IL-4, determined independently in four different laboratories (Powers et al., 1992; Smith et al., 1992; Walter et al.,

1992b; Wlodawer et al., 1992). Structures of other molecules with similar folds, for example, porcine (Abdel-Meguid et al., 1987) and human (de Vos et al., 1992) growth hormones, were also determined crystallographically. The structure of human growth hormone (HGH) was determined in a complex with the soluble part of its receptor (HGH-R). All of the cytokines listed above belong to the class of four-helix bundles with up-up-down-down topology, and structural similarities between them were noted previously (Diederichs et al., 1991; de Vos et al., 1992; Pandit et al., 1992; Wlodawer et al., 1992). Interferons β and γ also show general structural similarities to this class of cytokines, although an additional helix is found in interferon- β (Senda et al., 1992), and interferon- γ is a tight dimer in which each chain contains six helices. The helices that form the equivalent of the four-helix bundles discussed above come from both chains (Ealick et al., 1991; Samudzi et al., 1991).

Despite clear structural similarities between the hematopoietins, it was usually assumed that there is little if any direct similarity in their amino acid sequences, and, for example, the lack of such similarity between GM-CSF and IL-4 was explicitly mentioned (Powers et al., 1992; Smith et al., 1992). Limited similarity was noted previously, however, in the alignment of the amino acid sequences of human and murine IL-2 and GM-CSF (Schrader et al.,

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1986). The difficulties encountered in aligning the sequences of hematopoietins are not surprising because, in the absence of three-dimensional structures, alignment of the sequences of proteins for which residue identities are lower than 20–25% is usually difficult (Doolittle, 1985). Sequence alignment, however, was shown to have powerful predictive value for this system. Bazan (1992) showed, on the basis of structure-based sequence alignment of IL-4 and GM-CSF, as well as direct sequence alignment of IL-2 with the other two cytokines, that the originally published crystal structure of IL-2 (Brandhuber et al., 1987) could not be correct. Similar conclusions were also reached on the basis of structural studies of IL-2 utilizing the technique of multidimensional NMR (Mott et al., 1992). These suggestions have led to a significant modification of the IL-2 model (McKay, 1992), which is now in general agreement with the structures of other cytokines.

An interesting pattern of similarities between different cytokines is now emerging. Not only can clear similarity of the three-dimensional structures of these molecules be seen, but extracellular domains of the low-affinity subunits of their receptors belong to a single family sharing a related sequence (Bazan, 1990). It is very likely that the three-dimensional structure of the receptors, consisting of a duplicated motif of seven β strands and first observed in the complex with HGH (de Vos et al., 1992), is also conserved (Bazan, 1990). Under these circumstances, it is logical to postulate that the overall mode of binding between the ligands and the receptors might also be similar. If this is indeed the case, then the similarity of features in the surface areas of the cytokines involved in receptor binding might be related to providing the binding in the first place, while the differences might be important in establishing specificities. Assuming the existence of such similarities, the structure of the HGH/HGH-R complex (de Vos et al., 1992) provides an overall framework for the comparisons, and it was used here to a limited extent.

Because only C_{α} coordinates of the complex were available to us, no detailed comparisons involving the growth hormone were attempted. However, the refined structures of IL-2, IL-4, and GM-CSF are now available, allowing comparisons between them that involve not only the backbone but also amino acid side chains. We present such comparisons and correlate them with other available data, mostly obtained by mutation of surface residues in these cytokines.

Results and discussion

Superposition of atomic coordinates

Superimposed tracings of the C_{α} chains of IL-4, IL-2, and GM-CSF are shown in Figure 1 (see also Kinemage 1). It is clear that the areas of best superposition include primarily the four-helix bundle. As reported previously, however, the similarity is not limited to helical regions alone, but extends to some connecting loops as well, in particular to the loop following helix A and another preceding helix D (Wlodawer et al., 1992). For slightly more than half of the structure, based on C_{α} atom positions, the root mean square (rms) deviations fall within ~ 1.5 Å of each other. When analyzed in greater detail, the superpositions are not equally close for all helices, with the best agreement found between helices D of each structure (Table 1). It is also clear that although the positions of helices and the angles between them are quite well conserved, their lengths vary considerably from one structure to another.

To put the differences between these structures in another perspective, we compared the C_{α} coordinates of the X-ray (Wlodawer et al., 1992) and one of the NMR (Smith et al., 1992) models of IL-4. Their differences could be due to a combination of inaccuracies of each structure and genuine differences between the structures

Fig. 1. Stereo view of the superposition of the C_{α} traces of IL-4 (yellow), IL-2 (blue), and GM-CSF (red). All atoms in the side chains of cysteines forming disulfide bonds are shown as space-filling spheres.

Table 1. Deviations between atomic coordinates (in Å) for different structures of hematopoietins^a

	Individual molecules					
	IL-4/GM-CSF	IL-4/IL-2	IL-2/GM-CSF	IL-4 X-ray/NMR	IL-2	GM-CSF
A (4–16)	1.01	1.08	1.55	0.88	0.24	0.32
AB (22–30)	1.41	1.86	1.66	1.20	0.28	0.49
B (44–52)	1.83	1.17	1.31	0.80	0.20	0.22
C (83–95)	1.75	1.69	2.30	0.76	0.32	0.19
CD–D (107–120)	0.85	0.91	0.86	1.22	0.25	0.22

^a Structures are compared in the regions of structural similarity as defined in Figure 2. Residue numbering as for IL-4.

of proteins in crystals and in solution (Baldwin et al., 1991; Shaanan et al., 1992). For 126 out of 129 C α atoms, excluding the N-terminus, the rms deviations are 1.65 Å, whereas for the helical regions they range from 0.79 Å for helix C, 0.82 Å for helix A, and 0.97 Å for helix D to 1.78 Å for helix B. Comparisons with the high-resolution NMR structure of IL-4 (Powers et al., 1993) yield similar results. We may conclude that while some of the differences between the structures of the three cytokines might be due to the differences of crystal packing and to inaccuracies in structure determination, such influences are not major because they are on the same order as the differences found in the same structure studied by different means and in different environments. These differences are also much larger than those found in corresponding regions of the two crystallographically independent molecules of IL-2 (McKay, 1992), where the rms deviations are in the range of 0.2–0.3 Å (Table 1).

Sequence alignment

Structure-based alignment of the three amino acid sequences for those regions in which the three-dimensional structures are similar is shown in Figure 2. For IL-4 and GM-CSF, this alignment is identical to that of Bazan (1992), which was based on an X-ray model of GM-CSF (Diederichs et al., 1991) and on an NMR model of IL-4 (Smith et al., 1992). However, our alignment of IL-2 with the other two hematopoietins is not the same as that determined by Bazan (1992). In the latter alignment, the sequence in helix A is shifted by four residues in the direction of the C-terminus compared with the structure-based alignment, in the connecting region AB by two residues in the N-terminus direction, in helix B by five residues, and in helix C by three residues toward the C-terminus. Only helix D in the predicted model of IL-2 was aligned in a way that is in agreement with the three-dimensional structures. The alignment postulated by Schrader et al. (1986) does not agree with Figure 2 in any region, nor does it agree with the alignment of Bazan (1992). Because individual structural elements are shifted differently in the sequence-based alignments compared to the structure-based alignment presented here, it is not possible to postulate that another rigid superposition of IL-4

with other cytokines would better fit the previously published data.

This discrepancy could be due either to problems that remain with the crystal structure of IL-2 or to inaccurate alignment of its sequence. However, the positions of heavy atoms (McKay, pers. comm.) in relation to the current crystallographic model of IL-2 (McKay, 1992) strongly support its correctness. The two mercury sites are very

Fig. 2. Sequence alignment of human IL-2, IL-4, and GM-CSF. Filled rectangles mark those residues that are identical in IL-4 and either GM-CSF or IL-2, while gray rectangles mark residues that are similar in their properties. The similarity was considered to be either standard substitution of very similar residues (such as Ile for Leu) or preservation of structural features and other properties, such as hydrophobicity (e.g., for Phe and Leu). Residues that are expected to be involved in receptor binding, based on comparisons of these hematopoietins with human growth hormone, are marked with asterisks.

close to nitrogen atoms of His 16 of the two molecules in the asymmetric unit, while eight iodine sites are in the general vicinity of Tyr 31, Tyr 45, and Tyr 107 of both molecules. Any changes in the assignment of the sequence in line with the alignments presented by Bazan (1992) or Schrader et al. (1986) would destroy the correlations of the positions of the heavy atoms with their usual targets.

The identity and similarity of sequences resulting from different alignments are summarized in Table 2. For the purpose of this discussion, the residues are defined as being similar if their side chains are involved in similar types of interactions in the three-dimensional structure, rather than simply basing the similarity on the minimum base change of their codons. The regions of considerable structural similarity in the three three-dimensional structures (Fig. 1) are defined in Figure 2. As noted previously (Wlodawer et al., 1992), sequences of structurally aligned IL-4 and GM-CSF share some similarity, with 28% identity and 18% similarity for the 66 residues that belong to the highly conserved part of the three-dimensional structure and are listed in Figure 2. The percentages of identical and similar residues in IL-2 compared to IL-4 and to GM-CSF are summarized in Table 2. These data show that for alignment based on sequence alone, the fraction of *identical* residues may be similar or even higher than in structure-based alignment, but the percentage of matching *similar* residues is lower. The obvious reason is that structurally nonequivalent residues do not need to be homologous in general because their role in the three-dimensional structure of the protein might be different. Thus, the degree of similarity of residues (in addition to the identity) may be an indication of the correctness of the alignment based only on sequence similarity.

The results of the comparisons presented here point again to the difficulty of aligning sequences with low identity and intervening gaps, and caution against putting too much faith in the results of such alignments, which may yield correct overall conclusions while erring in details. The resulting low level of sequence similarity of residues after optimizing identities may be a warning of potential problems with the alignment. On the other hand, conclusions from structure-based similarities are more definitive (Jones et al., 1992). Nevertheless, caution should always be exercised if results of such predictions are used in

homology model building. Even though the overall conclusions of Bazan (1992) were correct, the sequence alignment on which they were based is not fully consistent with the corrected crystal structure of IL-2.

Disulfide bonds

The positions of disulfide bonds among the cytokines compared here are remarkably diverse (Fig. 1; Kine-mage 1). Human IL-4 has six cysteines forming three disulfide bridges, between residues 24 and 65, 46 and 99, and 3 and 127. The first pair connects two loop regions, AB and BC; the second pair is found between helix B and the loop CD; and the third connects the residue just preceding helix A with helix D. The latter disulfide is not conserved in mouse IL-4, where it is replaced by a bond between Cys 3 and Cys 92 (human numbering) (Carr et al., 1991). In this case, the covalent link is established between helices A and C rather than A and D, but this change is unlikely to require a major rearrangement in the structure.

Only two disulfide bonds are found in GM-CSF, linking residues 54–96 (N-terminus of helix B and N-terminus of β strand CD) and 88–121 (C-terminus of helix C and a strand following helix D). These pairings are conserved in GM-CSF from gibbon, bovine, and murine sources (Walter et al., 1992a). However, only a single disulfide bond between Cys 58 and Cys 105 is present in IL-2, connecting helix B to the strand CD. The integrity of this disulfide is required for biological activity (Ju et al., 1987). A free Cys 125, present in IL-2 and conserved throughout species (Bazan, 1992), does not have counterparts in GM-CSF or IL-4 and is not important to the function, because the alanine mutant used in crystal structure solution has identical activity (Brandhuber et al., 1987). None of these disulfides corresponds directly to the ones in IL-4, although Cys 121 in GM-CSF is a structural equivalent of Cys 127 in IL-4.

The only disulfide bridge that connects the same structural elements in the three cytokines is 46–99 in IL-4, which is reminiscent of the disulfide 54–96 in GM-CSF (although these disulfides do not superimpose very well in structural terms) and is found in the same area of the structure as 58–105 in IL-2. However, this disulfide appears to be most important in preserving the three-

Table 2. Identity and similarity of the residues in the three hematopoietins^a

	Proteins compared and comparison sources					
	IL-4/GM-CSF	IL-2/IL-4		IL-2/GM-CSF		
	Structure alignment	Structure alignment	Data of Bazan (1992)	Structure alignment	Data of Bazan (1992)	Data of Schrader et al. (1986)
Identity (%)	28	17	19.5	13.5	13.5	20
Similarity (%)	18	28	15	32	21	20

^a Structures are compared in the regions of structural similarity as defined in Figure 2.

dimensional structure of IL-4, with the mutants in which these residues were replaced by threonines losing 99% of activity (Kruse et al., 1991). It would be interesting to investigate whether or not a similar effect is also present in the other two cytokines.

The apparent lack of conservation of disulfides in four-helix bundle cytokines is in contrast to the situation observed for some cytokines with predominantly β -sheet structures. Daopin et al. (1992) showed that three disulfides present in β -nerve growth factor and transforming growth factor- β can be superimposed to within 1 Å, even though only two other residues were conserved in these two topologically related structures. Another predominantly β -sheet cytokine, platelet-derived growth factor, may also show a similar arrangement of disulfides, although detailed comparisons have not yet been presented (Oefner et al., 1992). It is clear that the role of the disulfides in preserving structural integrity of these two classes of cytokines must be completely different. For the fold that uses a hydrophobic core created by a four-helix bundle as the major structural component, stable structures may form quite independently of any covalent intrachain linkages. The cytokines in the β family, however, may rely on the presence of disulfide bridges to provide stability to otherwise very open structures.

Hydrophobic core

The hydrophobic core of the three cytokines compared here is primarily limited to the interior of the four-helix bundle (see Kinemage 2). However, some residues that do not belong to the helical regions also contribute their side chains to the core, for example, Thr 25, Val 29, Ile 32, and Phe 33 in IL-4. As expected, although the residues forming the core are predominantly hydrophobic, there is very little actual conservation of the sequence among the three cytokines. The only identical residues occupying similar positions in space are Leu 14, Leu 52, and Phe 112 (IL-4 numbering). Comparison of the cores shows clearly that the formation of the hydrophobic core in a four-helix bundle does not require strong similarity in the sequence of any individual helix. The hydrophobic core in different cytokines could be formed by the residues reaching the core from structurally nonequivalent positions or even from different helices, as long as the space inside the hydrophobic core is completely filled, maintaining continuous hydrophobic contacts. This type of behavior is common for mutants of proteins, where compensatory changes in hydrophobic cores are commonly observed (e.g., in HIV-1 and HIV-2 protease; Gustchina & Weber, 1991), and it is not surprising that it is also found among the distantly related cytokines. This phenomenon may explain the low level of sequence similarity for the class of proteins with highly conserved three-dimensional structure, given that the hydrophobic core includes more than half of the residues in a region with the conserved structure (Fig. 2), or about 30% of all of the residues in these proteins.

Helix A

The residues forming this helix show considerable degrees of both structural and sequence similarity among the three cytokines (Figs. 2, 3). Although some of the similarity and/or identity is found among residues forming the hydrophobic core, whose role thus should be limited only to maintaining three-dimensional structure, some of surface residues also show considerable conservation. Of particular interest are Gln 8 and Glu 9 in IL-4 (Kinemage 3), which correspond to Glu 20 and Glu 21 in human GM-CSF, whereas Lys 20 (human numbering) is present in mouse GM-CSF. The involvement of these residues in receptor binding in GM-CSF has been shown in a variety of studies. Shanafelt et al. (1991) have shown that Lys 20 of mouse GM-CSF is directly involved in binding to the β subunit of the receptor, and the nature of this residue provides species specificity. Lopez et al. (1992) have shown that mutation of Glu 21 in GM-CSF lowers or abolishes biological activity of the cytokine, again postulating its importance for binding to the β subunit. The structural equivalent of Glu 9 (IL-4) in HGH is Asn 12, which was shown to interact with the receptor (Table 2 in de Vos et al., 1992). No direct mutation data for equivalent residues are available at this time for IL-4, but the degree of conservation of the sequence suggests possible involvement of helix A in receptor binding of this cytokine as well (see below).

Site-directed mutagenesis experiments on IL-2 suggested that Asp 20 could play similar role for that cytokine (Zurawski et al., 1990; Sauv et al., 1991), and Imler et al. (1992) identified the region of the receptor involved directly in this interaction. That residue was aligned with Glu 21 in GM-CSF (Shanafelt et al., 1991). This alignment, however, does not correspond to structure-based superposition because the residue corresponding to Glu 21 is His 16, while Glu 15 in IL-4 corresponds to Gln 20 in GM-CSF and Gln 8 in IL-4 (Fig. 2).

Helices B and C

These two helices do not superimpose among the three cytokines equally as well as helices A and D, primarily due to the differences in interhelical angles. This is manifested by rms deviations of the C_α coordinates that are generally closer to 2 Å (especially for helix C) than to the ~1 Å observed for helices A and D (Table 2). Residue identities and similarities are largely restricted to hydrophobic side chains in the core of the four-helix bundle, although some surface residues are also conserved. Altmann et al. (1991) showed by proline substitution that the integrity of helix B is necessary for biological activity of murine GM-CSF, although the nature of that experiment says more about overall conformation than about specific interactions of individual residues. Shanafelt et al. (1991) have shown by deletion analysis that residues from helices B and C are important for biological activity of both human and murine GM-CSF, with critical residues being Thr 78

Fig. 3. Superposition of the residues forming parts of two most highly conserved helices, with conserved side chains shown explicitly. IL-4 is shown in yellow, IL-2 in blue, and GM-CSF in red. Residue numbering corresponds to the sequence of IL-4. Helix A is on the right, helix D on the left.

and Met 80 (human numbering). The latter residue is buried in the hydrophobic core, so the significance of this observation is unclear. Zurawski and Zurawski (1988) identified helix B of IL-2 as important to activity, while the region identified as helix B by Sauvé et al. (1991) was in reality the loop AB, and the misidentification was the result of the use of the obsolete model of Brandhuber et al. (1987).

An interesting structural feature is found on helix C in IL-4, and it appears to be unique to this cytokine. Five positively charged residues are located on that helix in the part that is longer than corresponding helices in the other two hematopoietins, with four (His 74, Arg 75, Arg 77, and Arg 81) pointing to the surface (Fig. 4). This positively charged row continues along the part of helix C that has structural equivalence in IL-2 and GM-CSF, encompassing Lys 84, Arg 85, and Arg 88. Together with Lys 12 from helix A, and Arg 53 and Lys 61 belonging to helix B, these residues make a very prominent positively charged patch completely exposed to solvent (Fig. 4; Kinemage 3). The importance of this feature for the biological activity of IL-4 and for its differentiation from other cytokines is unclear at the moment, but should be verified experimentally.

The importance of positively charged residues for biological activity of IL-2 was shown by Sauvé et al. (1991), who showed that residues Lys 35 and Arg 38 are involved in the receptor interactions. These residues are a part of

Fig. 4. Positively charged residues on the surface of IL-4. Three individual clusters are shown, as described in the text. The cluster at top left corresponds to the extension of helix C found in IL-4, the one at top right primarily to the conserved part of helix C, and the bottom cluster is made up of residues found on the C-terminus of helix D.

a patch on the top of the molecule, which also includes Lys 32, Lys 76, Arg 81, and Arg 83. Another positively charged patch is found on the bottom of the molecule and includes Lys 48, Lys 49, Lys 54, His 55, and Lys 97. The locations of these areas of positive charge are very different from those in IL-4 (discussed above).

Helix D

This helix and the connecting region CD are the most highly conserved structural elements among the three cytokines, both in terms of rms deviations of C α atoms (Table 1) and in terms of preservation of sequence (Figs. 2, 3). The general importance of helix D to the biological role of GM-CSF was noted by Shanafelt et al. (1991), although no specific residues were pointed out. However, specific parts of that helix were unambiguously shown to be crucial for the activity of both IL-4 and IL-2. Kruse et al. (1992) have converted IL-4 to an antagonist by mutating Tyr 124 to Asp, while mutation to Phe, His, Asn, Lys, or Gly resulted in partial agonists with unaltered receptor binding properties. Zurawski et al. (1990) have shown that substitutions of Gln 141 in IL-2 (mouse numbering, equivalent to Gln 126 in human IL-2) change the properties of this cytokine in a substantial way, affecting binding to the γ subunit of its receptor. These residues occupy positions on two different turns of the D helix, and the structural equivalent of Gln 126 in IL-2 is Arg 121 in IL-4. This, however, raises an interesting possibility, because by changing Tyr 124 to an acidic residue it is very likely that a salt bridge would be created between Arg 121 and Asp 124, thus the effect of this mutation might be less direct than originally postulated. This argument would be strengthened if an IL-4 mutant with Glu 124 would also be shown to be an antagonist.

Five positively charged residues are located on the C-terminus of helix D in IL-4, and four of them are exposed to solvent. These residues are Arg 115, Lys 117, Arg 121, and Lys 126 (Fig. 4; Kinemage 3). The participation of the end of helix D in receptor binding was reported for IL-4 (Postlethwaite & Seyer, 1991; Kruse et al., 1992), IL-2 (Zurawski & Zurawski, 1988; Zurawski et al., 1990), and GM-CSF (Diederichs et al., 1991; Shanafelt et al., 1991; Walter et al., 1992a). Positive charge is conserved at positions 115 and 117 (IL-4 numbering) in IL-2 and GM-CSF, respectively, and positively charged residues are found in HGH in positions structurally equivalent to residues 121 and 126 in IL-4, but nevertheless this patch appears to be a particular feature of IL-4. As mentioned above, similar arrangement of positively charged residues was also observed in helix C.

Models for cytokine-receptor interactions

The extracellular domains of cytokine receptors involved in the binding of hematopoietins share significant sequence and structural similarity (Bazan, 1990; Boulay & Paul,

1992). However, the only structure of an extracellular part of such a receptor that has been determined experimentally and published is from the complex of HGH with its receptor (de Vos et al., 1992). Each molecule of HGH is bound to two molecules of HGH-R, with a large part of the surface area of the hormone involved in the contacts with its receptors (Kinemage 4). This area consists of two sites: site I (in contact with molecule 1 of the receptor) and site II (contacting molecule 2). Site I is the major binding site, with the interacting region 25% larger than for site II. In addition, considerable interactions between the two receptor molecules are also introduced by their dimerization.

A high degree of structural similarity between IL-4 (and other cytokines for which three-dimensional structures are known) and HGH makes it possible to use the structure of the receptor in the complex of HGH/HGH-R to identify those areas of the other cytokines that are likely to be involved in the interactions with their respective receptors. For this purpose we superimposed the C α atoms of IL-4 and HGH, and found that this superposition does not lead to major steric clashes between IL-4 and the receptor molecules. We did not attempt to change the trace of the receptor to compensate for some insertions and deletions found when the sequences of HGH and IL-4 receptors are compared (Bazan, 1990).

Figure 5 shows the model of IL-4 interacting with two molecules of HGH receptor. By mapping those residues in IL-4 that are in close proximity to the receptor in this simple superposition, we can suggest that site I might include residues 18, 19, and 21 in helix A; 31, 34, and 36 in connecting loop AB; 105–108 in connecting loop CD; and 110, 111, 114, 115, 117, 118, 121, 122, 125, 126, and 129 in helix D. For site II, residues 6, 8, 9, 12, 15, and 16 in helix A and 74, 78, 81, 82, 85, and 88 in helix C could be involved in interactions with the extracellular domains of IL-4 receptors. It should be noted that ~85% of the residues mentioned above, marked with asterisks in Figure 2, belong to the regions with highly conserved three-dimensional structures in different cytokines, and some of them have similar or identical residues occupying structurally equivalent positions.

Comparison of the atomic models of the different cytokines discussed here shows clearly that different elements of their secondary structure vary in the degree of their conservation of both primary and tertiary structures. It might be reasonable to suggest that, for the parts of the extracellular domain of the cytokine receptor interacting with the highly conserved structural elements of its ligand, more similarity in binding could be expected between different species. In particular, the regions belonging to the C-terminal subdomains of the receptor (de Vos et al., 1992) involved in the interactions with helices D and A, where sequence and structure similarity is the highest, could retain considerable similarity in receptors for different cytokines. Conversely, less structure conservation is expected for the N-terminal subdomain of the receptor,

Fig. 5. Modeled interactions of IL-4 with its receptor. The model of IL-4 (yellow) was superimposed on the C_{α} model of human growth hormone (not shown). The unmodified trace of the human growth hormone receptor is shown in red.

which interacts either with those regions of cytokines in which conformations differ (such as connecting loops) or with the regions that have very specific features. One such region in IL-4 includes the concentration of positively charged residues on helix C, and another is found on the C-terminus of helix D, as described above (Fig. 4). Interactions involving these areas may be of particular impor-

tance because both of them involve the part of the receptor that includes Trp 104 in HGH-R (Fig. 6; Kine-mage 4), a residue that mediates contacts in both sites I and II and that plays an important role in ligand binding (Bass et al., 1991) but is not itself conserved in the IL-4 receptor. It must be stressed that both of these areas in IL-4 contain some similar features, namely a number of

Fig. 6. Helices C and D in IL-4 (yellow), with the side chains of positively charged residues on their surfaces shown in blue. Red loops correspond to the human growth hormone receptor in the vicinity of Trp 104, the latter marked with a dot.

positive charges on the surface (Fig. 6). An analysis of the area of the part of the IL-4 receptor that contains part of the sequence equivalent to Trp 104 in HGH-R (Bazan, 1990) shows the presence of several negatively charged residues. This indicates that electrostatic interactions may play an important role in the interactions in both sites I and II between IL-4 and its receptors, and explains the nature of different interactions between the same areas on the receptor but different parts of the ligand. One can also speculate that the positive patch on the surface of helix C might be important in attracting the second receptor molecule to the initial complex consisting of IL-4 and one receptor molecule only.

In the absence of experimental high-resolution crystal or NMR structures showing the interactions of different hematopoietins with their receptors, and with only limited mutagenesis data, the interpretation of the models based on similarity of primary through tertiary structures of the ligands must be accepted as only hypothetical. In particular, even in the regions where considerable structural similarity is preserved, it is possible that detailed interactions might differ due to compensatory changes of both the ligand and the receptor, as well as to the possibility of accomplishing similar binding utilizing residues that are not directly equivalent in their spatial location. This may indeed explain, for example, the apparent non-equivalency of Gln 126 in IL-2 with Tyr 124 in IL-4, despite some similarity of their biological roles.

However, attempts to map those regions of the cytokines that might be involved in the interactions with their receptors, as well as the regions on the receptors involved in cytokine binding, are quite reasonable. Similarities and differences discussed above may lead to designing experiments that could answer directly questions regarding the nature of such interactions and thus be of benefit in designing agonists and antagonists of these important signaling molecules.

Materials and methods

The coordinate sets used directly in all comparisons described here include the X-ray structures of IL-4 refined in our laboratory to $R = 0.218$ at 2.25 Å resolution (Wlodawer et al., 1992), GM-CSF refined to $R = 0.205$ at 2.4 Å (Diederichs et al., 1991), and IL-2 refined at 2.5 Å to $R = 0.202$ (McKay, 1992). The IL-4 model included all 129 residues found in the protein sequence, GM-CSF included 119 out of 127 residues (arbitrarily, only molecule 1 was considered in comparisons), and the model of IL-2 included 121 out of 133 residues (again only molecule 1 was used). In addition, coordinates of IL-4 resulting from the refinement of NMR data (Smith et al., 1992) were used in some comparisons, and the discussion of the interactions with the receptor was based on the C_α coordinates of the complex of HGH with its receptors (de Vos et al., 1992). All references to other cytokines are

only based on relevant publications and not on direct analysis of their coordinates.

Atomic models of the cytokines were displayed and manipulated on the Evans and Sutherland PS390 graphics terminal with the program FRODO (Jones, 1985). Superposition of the structures was accomplished for the most part with the program ALIGN (Satow et al., 1986), utilizing C_α coordinates only. In case of ambiguity, alignments were checked by hand and corrected using the RIGID option in FRODO. Figures were prepared with the program INSIGHT II, running on a Silicon Graphics Personal Iris 4D/35 computer.

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References

- Abdel-Meguid, S.S., Shiel, H.-S., Smith, W.W., Dayringer, H.E., Violand, B.N., & Bentle, L.A. (1987). Three-dimensional structure of a genetically engineered variant of porcine growth hormone. *Proc. Natl. Acad. Sci. USA* 84, 6434-6437.
- Altman, S.W., Johnson, G.D., & Prystowsky, M.B. (1991). Single proline substitutions in predicted α -helices of murine granulocyte-macrophage colony-stimulating factor result in a loss in bioactivity and altered glycosylation. *J. Biol. Chem.* 266, 5333-5341.
- Baldwin, E.T., Weber, I.T., St. Charles, R., Xuan, J.-C., Appella, E., Yamada, M., Matsushima, K., Edwards, B.F.P., Clore, G.M., Gronenborn, A.M., & Wlodawer, A. (1991). Crystal structure of interleukin-8: Symbiosis of NMR and crystallography. *Proc. Natl. Acad. Sci. USA* 88, 502-506.
- Bass, S.H., Mulkerrin, M.G., & Wells, J.A. (1991). A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. *Proc. Natl. Acad. Sci. USA* 88, 4498-4502.
- Bazan, J.F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* 87, 6934-6938.
- Bazan, J.F. (1992). Unraveling the structure of IL-2. *Science* 257, 410-412.
- Boulay, J.-L. & Paul, W.E. (1992). The interleukin-4 related lymphokines and their binding to hematopoietin receptors. *J. Biol. Chem.* 267, 20525-20528.
- Brandhuber, B.J., Boone, T., Kenney, W.C., & McKay, D.B. (1987). Three-dimensional structure of interleukin-2. *Science* 238, 1707-1709.
- Carr, C., Aykent, S., Kimack, N.M., & Levine, A.D. (1991). Disulfide assignments in recombinant mouse and human interleukin 4. *Biochemistry* 30, 1515-1523.
- Daopin, S., Cohen, G.H., & Davies, D. (1992). Structural similarity between transforming growth factor- β 2 and nerve growth factor. *Science* 258, 1161-1162.
- de Vos, A.M., Ultsch, M., & Kossiakoff, A.A. (1992). Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. *Science* 255, 306-312.
- Diederichs, K., Boone, T., & Karplus, P.A. (1991). Novel fold and pu-

- tative receptor binding site of granulocyte-macrophage colony-stimulating factor. *Science* 254, 1779-1782.
- Doolittle, R.F. (1985). Proteins. *Sci. Am.* 253(4), 88-99.
- Ealick, S.E., Cook, W.J., Vijay-Kumar, S., Carson, M., Nagabhushan, T.L., Trotta, P.P., & Bugg, C.E. (1991). Three-dimensional structure of recombinant human interferon- γ . *Science* 252, 698-702.
- Gustchina, A. & Weber, I.T. (1991). Comparative analysis of the sequences and structures of HIV-1 and HIV-2 proteases. *Proteins Struct. Funct. Genet.* 10, 325-339.
- Hill, C.P., Osslund, T.D., & Eisenberg, D. (1993). The structure of granulocyte-colony stimulating factor (G-CSF) and its relationship to those of other growth factors. *Proc. Natl. Acad. Sci. USA* 90, 5167-5171.
- Imler, J.-L., Miyajima, A., & Zurawski, G. (1992). Identification of three adjacent amino acids of interleukin-2 receptor β chain which control the affinity and the specificity of the interaction with interleukin-1. *EMBO J.* 11, 2047-2053.
- Jones, A. (1985). Interactive computer graphics: FRODO. *Methods Enzymol.* 115, 157-171.
- Jones, D.T., Taylor, W.R., & Thornton, J.M. (1992). A new approach to protein fold recognition. *Nature* 358, 86-89.
- Ju, G., Collins, L., Kaffka, K.L., Tsien, W.-H., Chizzonite, R., Crow, R., Bhatt, R., & Kilian, P.L. (1987). Structure-function analysis of human interleukin-2. *J. Biol. Chem.* 262, 5723-5731.
- Kruse, N., Lehrnbecher, T., & Sebald, W. (1991). Site-directed mutagenesis reveals the importance of disulfide bridges and aromatic residues for structure and proliferative activity of human interleukin-4. *FEBS Lett.* 286, 58-60.
- Kruse, N., Tony, H.-P., & Sebald, W. (1992). Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. *EMBO J.* 11, 3237-3244.
- Lopez, A.F., Shannon, M.F., Hercus, T., Nicola, N.A., Cambareri, B., Dottore, M., Layton, M.J., Elinton, L., & Vadas, M.A. (1992). Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. *EMBO J.* 11, 909-916.
- McKay, D.B. (1992). Unraveling the structure of IL-2. *Science* 257, 412-413.
- Milburn, M.V., Hassell, A.M., Lambert, M.H., Jordan, S.R., Proudfoot, A.E.I., Graber, P., & Wells, T.N.C. (1993). A novel dimer configuration revealed by the crystal structure at 2.4 Å resolution of human interleukin-5. *Nature* 363, 172-176.
- Mott, H.R., Driscoll, P.C., Boyd, J., Cooke, R.M., Weir, M.P., & Campbell, I.D. (1992). Secondary structure of human interleukin 2 from 3D heteronuclear NMR experiments. *Biochemistry* 31, 7741-7744.
- Nicola, N.A. (1989). Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem.* 58, 45-77.
- Oefner, C., D'Arcy, A., Winkler, F.K., Eggimann, B., & Hosang, M. (1992). Crystal structure of human platelet-derived growth factor BB. *EMBO J.* 11, 3921-3926.
- Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Kothe, K., & Kim, S.-H. (1992). Three-dimensional structure of dimeric human recombinant macrophage colony-stimulating factor. *Science* 258, 1358-1362.
- Postlethwaite, A.E. & Seyer, J.M. (1991). Fibroblast chemotaxis induction by human recombinant interleukin-4. *J. Clin. Invest.* 87, 2147-2152.
- Powers, R., Garrett, D.S., March, C.J., Frieden, E.A., Gronenborn, A.M., & Clore, G.M. (1992). Three-dimensional solution structure of human interleukin-4 by multidimensional heteronuclear magnetic resonance spectroscopy. *Science* 256, 1673-1677.
- Powers, R., Garrett, D.S., March, C.J., Frieden, E.A., Gronenborn, A.M., & Clore, G.M. (1993). The high-resolution three-dimensional solution structure of human interleukin-4 determined by multidimensional heteronuclear magnetic resonance spectroscopy. *Biochemistry* 32, 6744-6762.
- Samudzi, C.T., Burton, L.E., & Rubin, J.R. (1991). Crystal structure of recombinant rabbit interferon- γ at 2.7-Å resolution. *J. Biol. Chem.* 266, 21791-21797.
- Satow, Y., Cohen, G.H., Padlan, E.A., & Davies, D.A. (1986). Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7 Å. *J. Mol. Biol.* 190, 593-604.
- Sauvé, M., Nachman, M., Spence, C., Bailon, P., Campbell, E., Tsien, W.-H., Kondas, J.A., Hakimi, J., & Ju, G. (1991). Localization in human interleukin 2 of the binding site to the α chain (p55) of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA* 88, 4636-4640.
- Schrader, J.W., Ziltner, H.J., & Leslie, K.B. (1986). Structural homologies among the hematopoietins. *Proc. Natl. Acad. Sci. USA* 83, 2458-2462.
- Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K.T., & Mitsui, Y. (1992). Three-dimensional crystal structure of recombinant murine interferon- β . *EMBO J.* 11, 3193-3201.
- Shaanan, B., Gronenborn, A.M., Cohen, G.H., Gilliland, G.L., Veerapandian, B., Davies, D.R., & Clore, G.M. (1992). Combining experimental information from crystal and solution studies: Joint X-ray and NMR refinement. *Science* 257, 961-964.
- Shanafelt, A.B., Johnson, K.E., & Kastelein, R.A. (1991). Identification of critical amino acid residues in human and mouse granulocyte-macrophage colony-stimulating factor and their involvement in species specificity. *J. Biol. Chem.* 266, 13804-13810.
- Smith, L.J., Redfield, C., Boyd, J., Lawrence, G.M.P., Edwards, R.G., Smith, R.A.G., & Dobson, C.M. (1992). Human interleukin 4: The solution structure of a four-helix bundle protein. *J. Mol. Biol.* 224, 900-904.
- Walter, M.R., Cook, W.J., Ealick, S.E., Nagabhushan, T.L., Trotta, P.P., & Bugg, C.E. (1992a). Three-dimensional structure of recombinant human granulocyte-macrophage colony-stimulating factor. *J. Mol. Biol.* 224, 1075-1085.
- Walter, M.R., Cook, W.J., Zhao, B.G., Cameron, R.P., Ealick, S.E., Walter, R.L., Reichert, P., Nagabhushan, T.L., Trotta, P.P., & Bugg, C.E. (1992b). Crystal structure of recombinant human interleukin-4. *J. Biol. Chem.* 267, 20371-20376.
- Wlodawer, A., Pavlovsky, A., & Gustchina, A. (1992). Crystal structure of human recombinant interleukin-4 at 2.25 Å resolution. *FEBS Lett.* 309, 59-64.
- Zurawski, S.M., Imler, J.-L., & Zurawski, G. (1990). Partial agonist/antagonist mouse interleukin-2 proteins indicate that a third component of the receptor complex functions in signal transduction. *EMBO J.* 9, 3899-3905.
- Zurawski, S.M. & Zurawski, G. (1988). Identification of three critical regions within mouse interleukin-2 by fine structural deletion analysis. *EMBO J.* 7, 1061-1069.