Three-dimensional structures of α and β chemokines

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ABSTRACT **Members of the chemokine family of proteins play a key role in the orchestration of the immune response. This family has been further divided into two subfamilies, α and β, based on sequence, function, and chromosomal location. To date, the three-dimensional structures of two members of the α subfamily, interleukin-8 (IL-8) and platelet factor 4, and one member of the β subfamily, human macrophage inflammatory protein-1β (hMIP-1β), have been solved by either NMR or X-ray crystallography. In this review, we discuss their three-dimensional structures and their possible relationship to function. The structures of the monomers are very similar, as expected from the significant degree of sequence identity between these proteins. The quaternary structures of the α and β chemokines, however, are entirely distinct and the dimer interface is formed by a completely different set of residues. Whereas the IL-8 dimer is globular, the hMIP-1β dimer is elongated and cylindrical. Platelet factor 4 is a tetramer comprising a dimer of dimers of the IL-8 type. The IL-8 dimer comprises a six stranded anti-parallel β-sheet, three strands contributed by each subunit, on top of which lie two antiparallel helices separated by approximately 14 Å, and the symmetry axis is located between residues 26 and 26' (equivalent to residue 29 of hMIP-1β) at the center of strands β_1 and β_1'. In contrast, in the hMIP-1β dimer the symmetry axis is located between residues 10 and 10' which are part of an additional mini-antiparallel β-sheet formed by strands β_0 and β_0'; the two helices are 46 Å apart on opposite sides of the molecule; and strands β_1 and β_1' are about 30 Å apart and located on the exterior of the protein. Calculation of the solvation free energies of dimerization and analysis of hydrophobic clusters strongly suggests that the formation and stabilization of the two different types of dimers arise from the burial of hydrophobic residues, and that the distinct quaternary structures are preserved throughout the two subfamilies. The implications with regard to receptor recognition and the absence of cross-binding between the two subfamilies are discussed.—Clore, G. M., Gronenborn, A. M. Three-dimensional structures of α and β chemokines. *FASEB J.* 9, 57–62 (1995)**

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THE CHEMOKINE (CHEMOTACTIC CYTOKINE) superfamily of proteins plays a key role in the orchestration of the immune response, ensuring the correct immune effector cells are recruited and activated at the right place at the right time (1–5). These proteins comprise a polypeptide chain of ~8–10 kDa and contain four cysteine residues at near-identical positions. The superfamily has been further divided into two distinct subfamilies, α and β, based on whether the first two cysteine residues are separated by one residue (C-X-C) or are adjacent (C-C). This division fits well with the observation that the two subfamilies are encoded by distinct chromosomes, namely chromosomes 4 (q12–21) and 17 (q11–32) for the α and β chemokines, respectively. Members within each subfamily exhibit 25–70% sequence identity, while the amino acid identity between members of the two subfamilies ranges from 20 to 40%. The α chemokines (such as interleukin-8 [IL-8][2], GRO/MGSA, platelet factor-4 [PF4], NAP-II, and ENA-78) are potent chemoattractants and activators of neutrophils but not monocytes, whereas the β chemokines (such as macrophage inflammatory protein (MIP)-1β, MIP-1α, MCAF/MCP-1, RANTES, and I-309) exhibit chemoattractant potential for monocytes and lymphocytes but not neutrophils. The three-dimensional structures of two members of the α subfamily, IL-8 and PF4, and one member of the β subfamily, human MIP-1β (hMIP-1β) have been solved by NMR spectroscopy and/or crystallography (6–10). These structural studies revealed a highly unexpected finding, namely, while the tertiary structure of the chemokines were very similar, as expected from their high degree of sequence identity and similarity, the dimeric structures of members of the α and β subfamilies were completely different (10).

TERTIARY STRUCTURE

A superposition of the IL-8 and hMIP-1β monomers is shown in **Fig. 1A.** Within each subunit the main secondary structure elements comprise a triple-stranded antiparallel β-sheet arranged in a Greek key, on top of which lies a long C-terminal α-helix (6, 10). The N-terminus comprises an irregular strand and a series of non-classical turns which form a long loop extending to a short helical turn which leads into strand β_1. The C^α atoms of 59 residues of IL-8 and hMIP-1β can be superimposed with an rms difference of 1.6 Å. The sequence identity within this region is 20% (Fig. 1B). Moreover, residues that are buried within the core of the monomer and have surface accessibilities of less than 20% of the equivalent amino acid in a Gly-X-Gly tripeptide are either the same or substituted conservatively (Fig. 1B). Of the 12 identical residues between the two proteins, eight have surface accessibilities less than 30% of that in a Gly-X-Gly tripeptide, and, except for the conserved cysteines, none are located at the dimer interface of the two proteins (see below).

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[2]Abbreviations: IL-8, interleukin-8; MIP, macrophage inflammatory protein; hMIP-1β, human MIP-1β; PF4, platelet factor-4; SFED, solvation free energy of dimerization.

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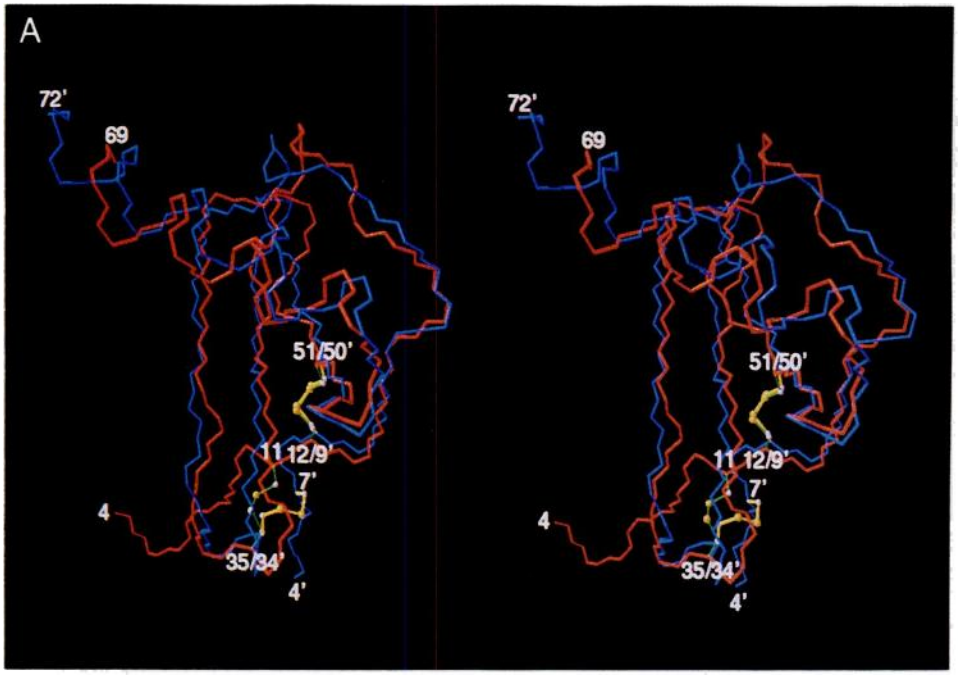
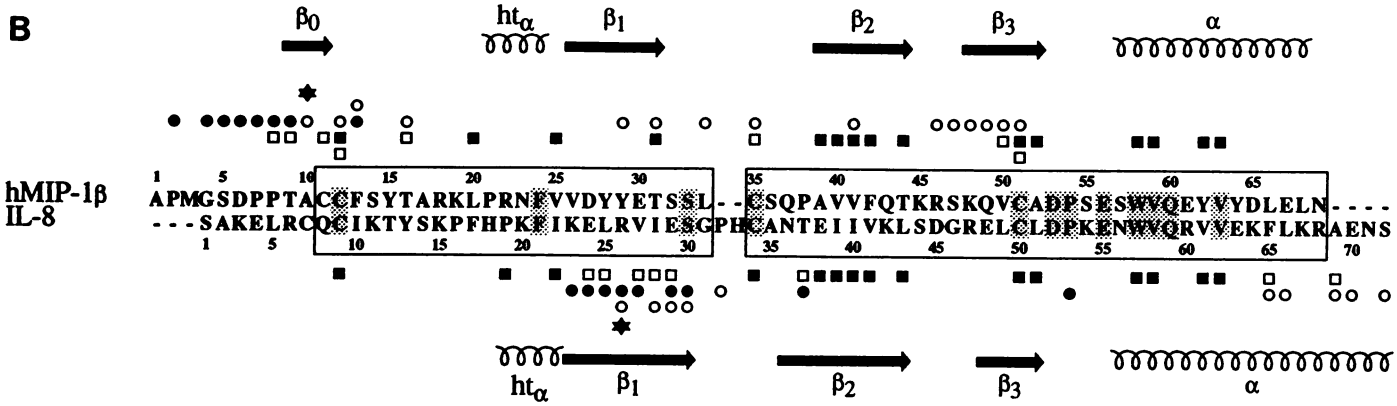


Figure 1. *A)* Stereoview showing a best-fit superposition of the backbone atoms and disulfide bridges of a single subunit of hMIP-1β (red with disulfides in green) and IL-8 (blue with disulfides in yellow). *B)* Sequence alignment of hMIP-1β and IL-8 based on the structure alignment shown in (*A*) together with the location of the secondary structure elements. The sequences that are structurally aligned in (*A*) with a C^{α} atomic rms difference of 1.6 Å are boxed; residues that are identical between hMIP-1β and IL-8 are shaded; the filled-in squares above or below the sequence represent residues in each monomer with a surface accessibility of <20% of that in an isolated Gly-X-Gly extended tripeptide (29); the open squares represent residues which are buried upon dimerization and have a surface accessibility of <20% in the dimer; when both an open and a filled-in square are shown, the surface accessibility of that particular residue is not only <20% in the monomer but its surface accessibility is also decreased by more than a factor of 7 in the dimer; the filled-in and open circles indicate the residues of subunit A that interact with those of subunit B, respectively, and vice versa; the stars indicate the location of the C_2 symmetry axis for each dimer. From ref 10.



This indicates that the identical residues form internal contacts that are necessary to maintain the monomer structure, but that the surface residues necessary for dimer formation and receptor binding can vary between the two subfamilies of chemokines (10).

There are, however, four significant structural differences between IL-8 and hMIP-1β at the monomer level (10). First, the conformation of the first disulfide bridge is a right handed hook in IL-8 as opposed to a left-handed spiral in hMIP-1β (Fig. 1A). This is associated with the insertion in IL-8 of a residue between the first two cysteines and of two residues in the turn connecting β-strands 1 and 2 (Fig. 1B). In contrast, the second disulfide bridge is almost perfectly superimposable between the two structures (Fig. 1A). Second, the helix extends for five residues further at the C-terminus in IL-8 compared to hMIP-1β. Third, the conformation of the turn connecting strands β_2 and β_3 differs around residues 46 and 47 (residue number of hMIP-1β). Finally, the direction of the N-terminal residues preceding the first cysteine is completely different. The latter three differences at the monomer level are related to the different quaternary structures of the two proteins, as will be discussed below.

QUATERNARY STRUCTURE

In contrast to their similarity at the monomer level, the dimer structures of hMIP-1β and IL-8 are entirely distinct, and the interface is formed by a completely different set of residues. Note that the quaternary structure of the other α chemokine whose structure has been solved, PF4, is a tetramer formed by a dimer of dimers of the IL-8 type (9). The difference in dimeric structure is readily appreciated from the ribbon diagrams presented in **Fig. 2** which show a view of the IL-8 dimer (Fig. 2A), a view of the hMIP-1β dimer (Fig. 2B), and a superposition of the two dimers (Fig. 2C). In the latter, one subunit of hMIP-1β has been superimposed on one subunit of IL-8, and the C^{α} atomic rms displacement between the second subunit of hMIP-1β and the second subunit of IL-8 is 34 Å. Different quaternary structures for essentially identical monomer units are clearly very rare occurrences and to our knowledge have been observed in cases with significant sequence identity (that is greater than 15–20%) on only two previous occasions (11, 12).

The IL-8 dimer is globular in shape with dimensions of 40 × 42 × 32 Å (Fig. 2A), while the hMIP-1β dimer is elongated and cylindrical with dimensions of 56 × 30 ×

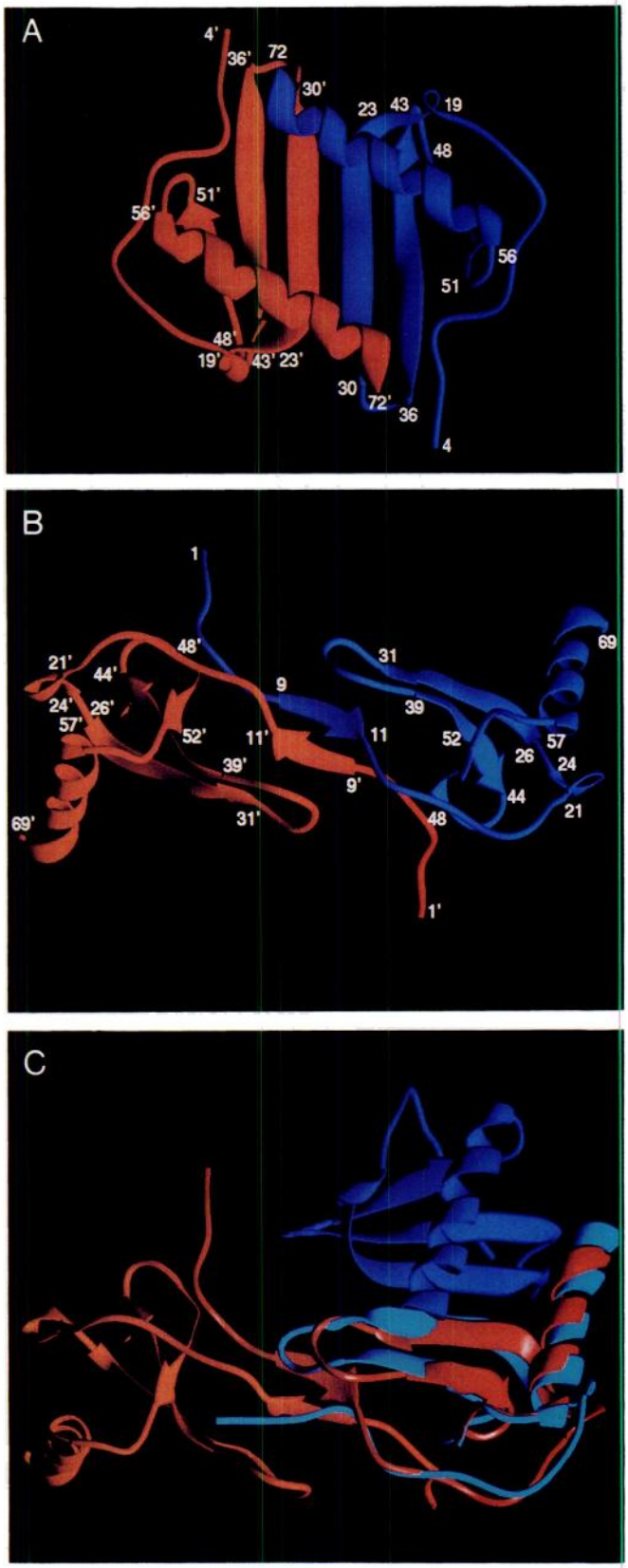


Figure 2. Schematic ribbon drawings of the IL-8 dimer (*A*), the hMIP-1β dimer (*B*), and a superposition of the hMIP-1β and IL-8 dimers (C). In (*A*) and (*B*) one subunit is shown in blue and the other in red; in (C) the hMIP-1β subunits are shown in red and orange while the IL-8 subunits are shown in light blue and blue, with the red subunit of hMIP-1β superimposed on the light blue subunit of IL-8 using the same alignment as in Fig. 1. From ref 10.

26 Å (Fig. 2B). This is reflected in the very different ratios of the three principal components of the inertia tensor, namely, 1.0:0.92:1.23 for IL-8 compared to 1.0:3.72:3.84 for hMIP-1β. In the case of IL-8, the C_2 axis is located between the $C^{\alpha}H$ protons of Arg26 and Arg26' in IL-8 (equivalent to residue 29 of hMIP-1β), and the dimer interface comprises an antiparallel β-sheet in which strand β_1 of one subunit is hydrogen bonded to strand β_1' of the other (Figs. 2A and C). This results in a structure with a six-stranded antiparallel β-sheet, three from each subunit, on top of which lie two antiparallel α-helices separated by approximately 14 Å. In addition, the C-terminal end of the helix of each subunit interacts with the underlying sheet of the other subunit (Figs. 1B and 2A).

In contrast, in the case of hMIP-1β, the C_2 axis is located between the $C^{\alpha}H$ protons of Ala10 and Ala10'; the two helices are 46 Å apart, located on opposite faces of the molecule, and oriented approximately orthogonal to each other; strand β_1 and β_1' are ~30 Å apart and located on the exterior of the protein; and the dimer interface is formed by the N-terminus (residues 2–13), the loop connecting strands β_1 and β_2 (Leu34 and Cys35), and the loop connecting strands β_2 and β_3 as well as strand β_3 (residues 46–51) (Figs. 1B, 2B, and 2C). A comparison of the residues involved in the dimer interfaces of hMIP-1β and IL-8 is provided in Fig. 1B.

The hydrogen bonding interactions stabilizing the IL-8 dimer are restricted to the six backbone hydrogen bonds between strand β_1 of one subunit and strand β_1' of the other (6). In the case of hMIP-1β, there are eight intersubunit backbone hydrogen bonds (10). Four of the hydrogen bonds make up a small antiparallel β-sheet centered around the C_2 axis which comprises residues 9–11 and 9'–11' (strands β_0 and β_0'). In addition Asp6(NH) donates a hydrogen bond to Gln49'(O), while Asp6(O) accepts a hydrogen bond from Cys51'(NH). Despite the extended nature of the hMIP-1β dimer, there is an extensive network of hydrophobic interactions between the two subunits, and 8 residues per subunit, the same number as in IL-8, become buried (as defined by a surface accessibility of $<20\%$ compared to that in a Gly-X-Gly tripeptide) upon dimerization (Fig. 1B).

What is the structural basis for the dramatic difference in the IL-8 and hMIP-1β? Apart from backbone hydrogen bonding, all the interactions that stabilize the dimer in hMIP-1β are hydrophobic in nature, and all but one, consisting of a potential single salt bridge, are hydrophobic in IL-8.

To examine this question we proceeded to calculate the solvation free energy of dimerization (SFED; ref. 13) of hMIP-1β and IL-8 monomers to both the hMIP-1β and IL-8 type dimers (10). Dimerization of hMIP-1β monomers to the hMIP-1β and IL-8 type dimers yields SFED values of -12.5 and -3.3 kcal.mol^{-1}. Thus, in the case of hMIP-1β, the hMIP-1β type dimer is favored by -9.2 kcal.mol^{-1} over the IL-8 type dimer. In contrast, dimerization of IL-8 monomers to the hMIP-1β and IL-8 type dimers yields SFED values of -4.6 and -7.0 kcal.mol^{-1}. Thus, in the case of IL-8, the IL-8 type dimer is favored by -2.4 kcal.mol^{-1} over the hMIP-1β type dimer. These calculations strongly support the proposition that the driving force for the formation and stabilization of the two different types of dimers lies in the burial of hydrophobic residues. In the case of hMIP-1β, these are buried more effectively for the hMIP-1β type dimer than for the IL-8 type dimer, while the converse holds for IL-8.

Examination of the structure based sequence alignment of hMIP-1β and IL-8 shown in Fig. 1B yields a possible explanation for this phenomenon. First, IL-8 has an extra four

residues at the C-terminus that permit extension of the helix onto the adjacent subunit, helping to form the IL-8 dimer. In hMIP-1β, on the other hand, the helix cannot extend beyond the boundaries of its own subunit. Second, in the IL-8 dimer the residues that point upwards into the cleft (Leu25 and Val27) and across the center of the cleft (Leu66) formed by the two helices are hydrophobic. In contrast, the equivalent three residues in hMIP-1β are polar (Tyr28, Glu30 and Glu67, respectively). The concentration of four partially buried negative charges in very close proximity provided by Glu30 and Glu67 of each subunit would, on the basis of simple electrostatic considerations, disfavor the formation by hMIP-1β of an IL-8 type dimer. Third, the presence of three proline residues renders the N-terminus of hMIP-1β predominantly hydrophobic, whereas this region is mainly polar in IL-8. Thus, the formation of the hMIP-1β dimer permits burial of these hydrophobic residues.

In this regard, it is also of interest to examine the sequences of the other members of the α and β chemokine subfamilies (10). In general, the β chemokines have fewer residues at the C-terminus than the α chemokines. Exceptions to this are MCAF/MCP-1 and mJE in the β subfamily and human PF4 in the α subfamily. The sequences N-terminal to the first cysteine residue are always more hydrophobic in the members of the β subfamily than those in the α subfamily. Finally, residues that correspond to Leu25, Val27, and Leu66 in IL-8, which are crucial in stabilization of the IL-8 dimer, are always hydrophobic in the α subfamily and always polar in the β subfamily. Specifically, Leu25 is always replaced by a Tyr, and Val27 and Leu66 are usually substituted by a charged residue. (Val27 is substituted by Glu, Arg, or Lys except in the case of RANTES where it is substituted by Tyr, and Leu66 is substituted by Asp or Glu except in the case of mcTA3 where it is substituted by Asn). This suggests that the IL-8 and hMIP-1β dimer structures are preserved in the α and β subfamilies, respectively.

The same conclusions have been reached by a quantitative cluster analysis of the hydrophobic properties of α and β chemokine sequences, based on the coordinates of IL-8 and hMIP-1β (14). Thus, we find that the monomers of the α and β chemokines have their strongest hydrophobic cluster at equivalent positions within the protein core consistent with their similar tertiary structures. In contrast, the patterns of monomer surface hydrophobicity between the α and β chemokines differ in a manner that is fully consistent with the observed differences in quaternary structure between IL-8 and hMIP-1β. The most hydrophobic surface clusters on the monomer subunits are located in very different regions of the α and β chemokines and comprise in each case the amino acids that are buried at the interface of their respective dimers.

In the light of these results it is likely that the proposed dimeric structure of the β chemokine MCAF/MCP-1, which we had previously modeled on the basis of the IL-8 dimer, is incorrect (15). This only serves to emphasize the importance of direct experimental structure determination, particularly in the case of multimeric proteins.

LOCATION OF RECEPTOR BINDING SITES

The topology of the IL-8 dimer is remarkably similar to that of the α1/α2 domains of the Class I major histocompatibility antigen HLA-A2 (16). This led to the initial suggestion of a functional role for the two antiparallel helices and the cleft between them in binding to the IL-8 receptor (6). Extensive mutagenesis data, however, has yielded no support for this hypothesis, and it now seems likely that the two helices are probably involved in binding to proteoglycan on the surface of the endothelium, thereby presenting IL-8 to circulating neutrophils on a solid support (1–5). Mutagenesis studies of IL-8, however, have identified the N-terminus, Glu4-Leu5-Arg6 (ELR) and Ile10, as important in receptor binding and cell activation (17, 18). Interestingly, the first five residues of IL-8 are disordered in solution (6) and the first three in the X-ray structure (7). Further, the ELR sequence cannot be the only structurally pertinent region of IL-8 since GRO/MGSA contains the ELR sequence but inhibits IL-8 binding to the Type 2 receptor only (19). Moreover, IL-8 analogs shortened at the C-terminus show progressively reduced potency as residues are deleted (20).

Recently, we used heteronuclear NMR spectroscopy to map the binding surface on IL-8 for a N-terminal fragment (residues 1–40) of the human Type-1 IL-8 receptor (21). Although this peptide binds very weakly (K_D ~170 ± 50 μM), it selectively perturbs the chemical environment of a number of residues, located in strand β_3 of the β-sheet (Glu48 to Cys50), the turn preceding β_3 (Ser 44), the C-terminal α-helix (Val61), and the irregular N-terminal loop region (Thr12, Lys15, Phe17, His18, Lys20, and Phe21). This is depicted in a schematic ribbon drawing of IL-8 in **Fig. 3.** Thus, the IL-8 dimer contains two binding areas for the peptide, rather than one contiguous surface, suggesting that two peptides bind per dimer, each one sliding into the cleft between strand β_3 and the 12–21 loop. Since the N-terminal fragment of the IL-8 receptor is acidic (9 of 40 residues are aspartic or glutamic acids), basic residues of IL-8 may be involved in the interaction. Inspection of the region highlighted in Fig. 3 indicates that Lys11, Lys15, Lys20, Lys42, and Arg47 are plausible candidates for this. In addition, Tyr13, Phe21, and Leu49 may be involved in hydrophobic contacts with the receptor. Clearly these results should be interpreted with some degree of caution as the peptide/protein model system studied lacks several key features of the intact IL-8/IL-8 receptor system, including

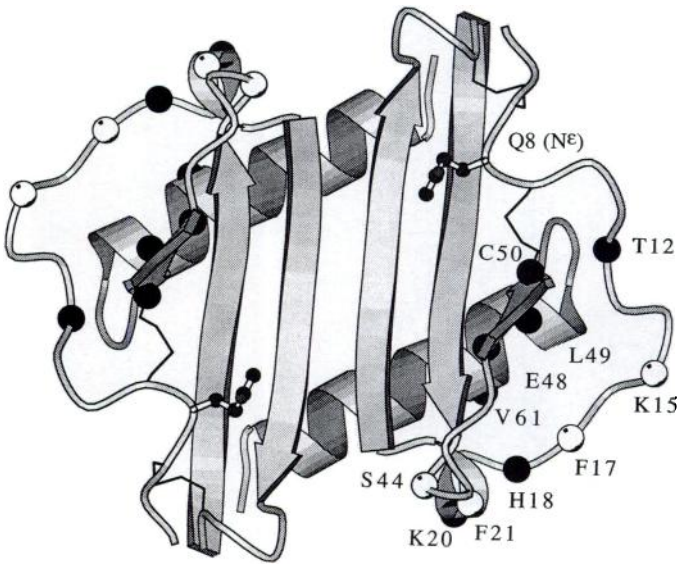


Figure 3. Schematic ribbon diagram of the solution structure of the IL-8 dimer. The C^{α} atoms of residues that exhibit the largest ^{15}N (>10.49 ppm) and ^{1}H (>10.1 ppm) chemical shift changes upon titration with a 40-residue peptide comprising the extracellular N-terminal fragment of the human Type-I IL-8 receptor are denoted with black and white spheres, respectively. From ref 21.

extracellular loops of the IL-8 receptor, N-glycosylation of residues at its N-terminus, and the unique local environment present at the surface of the membrane.

In the case of hMIP-1β we have postulated that the large concave surface visible in **Fig. 4,** which runs at approximately 60° to the long axis of the dimer, is involved in receptor binding of the β chemokines (10). There are two residues on the concave surface of the dimer that are identical in hMIP-1β, MCP-1/MCAF, hMIP-1α, and RANTES (Tyr28, Ser32). Further, Pro8, Ala10, Asp27, and Glu67 are either the same or substituted conservatively. All six residues are indicated in green in Fig. 4, and we suggest that they play an important role in binding specificity. Support for this suggestion comes from the observation that mutation of Tyr28 to Asp in MCP-1/MCAF abolishes monocyte chemoattractant activity, and that mutation of Asp6 (which is equivalent to Glu67 of hMIP-1β) to Leu in MCP-1/MCAF generates a protein with only 10–20% of wild type activity (22). Of the four β chemokines only hMIP-1α and RANTES induce a strong calcium flux in the cloned receptor (23, 24). This activity may be associated with the four residues (shown in orange in Fig. 4) in the cleft, which are preserved in hMIP-1α and RANTES but are different in hMIP-1β or MCAF/MCP-1.

CONCLUDING REMARKS

Structural studies reveal that while the monomeric structures of the α and β chemokines are very similar, their dimeric structures, as typified by IL-8 and hMIP-1β, respectively, are dramatically different. Since those features of IL-8 and hMIP-1β that are responsible for their different dimeric structures are preserved throughout the α and β subfamilies respectively, we predict that other members of the α and β subfamilies will also form similar dimeric structures as those formed by IL-8 and hMIP-1β, respectively. This provides an elegant explanation for the lack of receptor cross-binding and reactivity between the α and β chemokine families. The biological significance of the dimeric form of the chemokines, however, remains to be established. A recent study in which the backbone amide of Arg26 of IL-8 was replaced by a N-methyl group yielded a monomeric form of IL-8 that was still active (25). In addition, the circulating concentrations of chemokines (in the nM range) are well below the equilibrium constant for dimerization ($\sim$0.1 μM) so that freely circulating chemokines would be monomeric. However, there is good indication that the chemokines act in an immobilized form bound to the endothelial surface, thereby preventing the soluble chemokines from being washed away rapidly in vivo and permitting the formation of a chemokine concentration gradient down which the effector cells (lymphocytes, neutrophils, monocytes) can migrate (26). Under these conditions, the local concentration of chemokine would be high and the dimeric form would predominate. Finally, evolutionary considerations also suggest that the dimeric form is important. The formation of a distinct and stable dimer requires the presence of a specific cluster of residues on the surface of the monomer capable of forming a compementary dimer interface. If the dimeric form were unimportant, there would be no evolutionary pressure to conserve the interacting residues located at the dimer interface. FJ

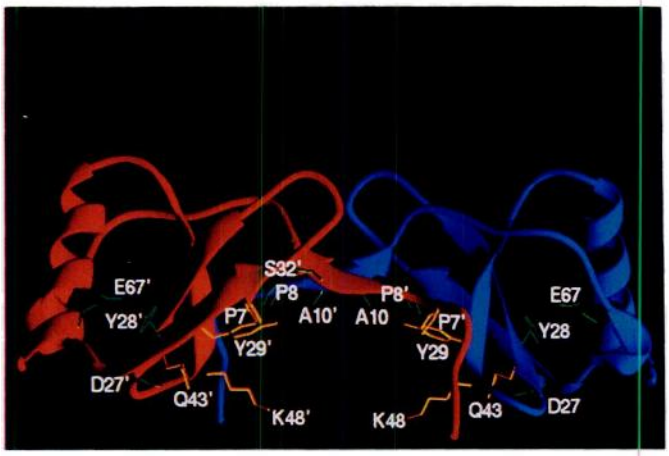


Figure 4. Ribbon diagram of hMIP-1β showing side chains within the concave cleft that may be involved in receptor binding; side chains shown in green are either the same or substituted conservatively in hMIP-1β, hMIP-1α, RANTES, and MCAF/MCP-1, while side chains shown in orange are the same or similar in hMIP-1α and RANTES but different in hMIP-1β and MCAF/MCP-1. From ref 10.

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