The Structure of Human Macrophage Inflammatory Protein-3α/CCL20

LINKING ANTIMICROBIAL AND CC CHEMOKINE RECEPTOR-6-BINDING ACTIVITIES WITH HUMAN β-DEFENSINS*

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Human macrophage inflammatory protein-3α (MIP-3α; CCL20) is a CC-type chemokine that binds to and activates CC chemokine receptor-6 (CCR6). Although MIP-3α does not share the binding site of CCR6 with any other chemokine, human β-defensin-1 and -2, small cationic antimicrobial peptides, have also been found to bind to and activate CCR6. Conversely, we have found that MIP-3α possesses antibacterial activity of greater potency than human β-defensin-1 and -2 against Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213, while having no activity against the fungus Candida albicans. There is no clear sequence similarity between β-defensins and the chemokine MIP-3α, beyond an abundance of cationic residues and the presence of disulfide bonds. Nonetheless, there are structural similarities between these three proteins that allow their overlap of chemotactic and antimicrobial activities. In this report, we describe the x-ray crystal structure of human MIP-3α refined to a resolution of 1.7 Å and compare it with the crystal structures of human β-defensin-1 and -2. Molecules of MIP-3α and the β-defensins seem to share few structural motifs that are likely associated with their common biological activities.

Chemokines constitute a family of small (5–20 kDa), positively charged, chemotactic cytokines that regulate immune responses by binding to seven-transmembrane G-protein-coupled chemokine receptors on the surface of leukocytes (1). Currently, there are nearly 50 chemokines and at least 20 chemokine receptors identified in humans (2, 3). Chemokines are characterized on the basis of a pattern of conserved cysteine residues near the N terminus and have been classified into four subfamilies; C, CC, CXC, and CX3C (2). Chemokines are secreted and immobilized on the surface of cells, where they provide a chemotactic gradient directing leukocytes toward the site of inflammation. The binding of chemokines to and activation of their cognate receptors cause an increase in integrin adhesiveness on the cells expressing the receptors, stable arrest, and migration of the cells along the chemokine gradient (4). Beyond this primary function, chemokines also play a part in angiogenesis/angiostasis, hematopoiesis, metastasis, and lymphatic organogenesis (1).

The chemokine macrophage inflammatory protein-3α (MIP-3α; LARC/Exodus-1/CCL20) is a 9-kDa CC-type chemokine that was identified by means of bioinformatics (5). MIP-3α is expressed constitutively by keratinocytes in epidermal layers of the skin (6, 7), intestinal mucosa (8), epithelial crypts of tonsils (9), and the epithelium of Peyer’s patches in the intestine (10, 11). Murine MIP-3α is expressed in the thymus, small intestine, and colon (12). Although many chemokines and chemokine receptors are promiscuous in their interactions, MIP-3α is the sole chemokine ligand of its receptor, CC chemokine receptor-6 (CCR6) (13, 14).

MIP-3α attracts natural killer cells and memory T-cells to the site of inflammation, similar to the innate immune response of other chemokines (15, 16). However, MIP-3α triggers the start of an adaptive immune response primarily by attracting immature dendritic cells expressing CCR6 on their surfaces to the site of inflammation, allowing the dendritic cell to take up a foreign antigen and to mature (4, 9). After binding MIP-3α, the expression of CCR6 is down-regulated, causing the dendritic cell to migrate to the lymph nodes, where it binds via CCR7 to MIP-3β and presents the antigen to CD8+ T-cells (17). In this way, MIP-3α can bridge the innate and adaptive immune response. MIP-3α also plays a role in psoriasis (6) and liver disease (18) and may be useful in treating various autoimmune disorders (19–23).

Although MIP-3α is the only chemokine ligand to CCR6, two human β-defensins, β-defensin-1 (hBD1) and β-defensin-2 (hBD2), were shown to also bind to and activate CCR6 (24). Defensins are small (3–5 kDa), highly positively charged peptides whose topology is stabilized by three intramolecular disulfide bonds and primarily serve as antimicrobial agents (25). Both hBD1 and hBD2 have no discernible amino acid sequence similarity.
similarity to MIP-3α (Fig. 1), and it has been postulated that their overlapping activities are entirely dependent on the similarity of their three-dimensional structures and localization of their charges (26). The high-resolution structures of hBD1 and hBD2 were characterized in great detail (27, 28). A comparative analysis of the three structures would provide insight into the nature of not only the CCR6-binding site, but also antimicrobial activity, by showing elements of similarity between the structures. Here, we present the high-resolution crystal structure of human MIP-3α refined at 1.7 Å. We also present evidence that human MIP-3α has potent antimicrobial activity. Furthermore, the receptor-binding and antimicrobial activities shared between β-defensins and MIP-3α are described in terms of structural and electrostatic overlap.

**EXPERIMENTAL PROCEDURES**

**Protein Synthesis, Purification, and Refolding**—Machine-assisted stepwise solid-phase peptide synthesis was carried out on an Applied Biosystems Model 433A synthesizer using a custom-designed program tailored from the published in situ neutralization/HBTU activation protocol for t-butyloxycarbonyl chemistry (29). The amino acid sequence of MIP-3α is as follows: Asn(2-tosyl)-Lys(2-chloro-benzyloxycarbonyl), Ser(benzyl), Thr(benzyl), GCDINAIIFH TKKKLSVCAN PKQTWVKYIV RLLSKKVKNM. The following side chain protection was used: Arg(tosyl), Asn(xanthyl), GCDINAIIFH TKKKLSVCAN PKQTWVKYIV RLLSKKVKNM. The migration of CCR6-transfected HEK293 cells was observed by immunofluorescence microscopy.

**Chemotaxis Assay**—The migration of CCR6-transfected HEK293 cells was observed by immunofluorescence microscopy.

**Data and refinement statistics**

| Parameter                      | Value  |
|--------------------------------|--------|
| Wavelength (Å)                 | 0.98   |
| Space group                    | P6₁    |
| Unit cell (Å)                  | a = b = 70.82, c = 71.77 |
| Resolution range (Å)<sup>a</sup> | 20.0 to 1.70 (1.76 to 1.70) |
| R<sub>merge</sub><sup>a,b</sup> | 0.087 (0.620) |
| Total no. of observations      | 99,101 |
| No. of independent observations| 21,953 |
| Completeness (%)<sup>c</sup>   | 97.5 (96.8) |
| Average I/ΔI<sup>d</sup>       | 23.8 (1.9) |
| No. of reflections             | 19,365 |
| Working set                    | 19,365 |
| Test set                       | 834    |
| Resolution range (Å)           | 15.0 to 1.70 |
| R<sub>merge</sub><sup>c</sup>  | 0.176/0.217 |
| No of all non-H atoms          | 1231   |
| Water molecules                | 169    |
| Heterogen atoms                | 40     |
| Average B-factor (Å<sup>2</sup>)| 27.7   |
| Protein atoms                  | 26.5   |
| No. of disordered residues     | 5      |
| r.m.s.d.<sup>e</sup> from ideality | 0.007 |
| Bonds (Å)                      | 0.034  |
| Angle distances (Å)            | 0.028  |
| Plane distances (Å)            | 0.028  |

<sup>a</sup> The highest resolution shell ranges are shown in parentheses.
<sup>b</sup> R<sub>merge</sub> = Σ[I(λ) - I(λ')]/Σ[I(λ)].
<sup>c</sup> R<sub>merge</sub> = Σ[I(λ)]/[Σ[I(λ')]].
<sup>d</sup> R<sub>merge</sub> = Σ[I(λ)]/[Σ[I(λ')]], where T represents a test set of reflexions (4% of the total, chosen at random, not used in the refinement).
<sup>e</sup> Root mean square deviation.

**Structure Solution and Refinement**—The phases were obtained by molecular replacement using AMoRe (32). Based on the calculated molecular volume (33), it was assumed that there would be two to three molecules in the asymmetric unit. Searches using the model based on the x-ray structure of Met-RANTES (regulated on activation normal T cell expressed and secreted) (34) in the resolution range 12 to 2.8 Å were successful in locating the positions of two molecules within the asymmetric unit. This was somewhat unexpected based on the calculated relatively high solvent content (62%) and the quality of diffraction.

The model was rebuilt based on the amino acid sequence of human MIP-3α and refined using the program CNS (35), with the resolution gradually extended to the range 8.0 to 1.8 Å. Some fragments of the model were corrected manually using the program O (36), and many solvent sites were located. Multiple isopropyl alcohol molecules were also identified. Further refinement to 15.0 to 1.7 Å was done using the program SHELXL (37). At the last stages of refinement, anisotropic displacement parameters were applied to sulfur atoms (eight cysteines). The final model consists of protein residues 5–65 in two independent monomers, 169 water molecules, and 10 isopropyl alcohol molecules. The R value for all reflections (15.0 to 1.7 Å) is 19.0% (R<sub>free</sub> = 23.9%).

**Sedimentation Equilibrium**—Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A ultracentrifuge using a Ti-60 rotor and double-sector aluminum centerpieces (optical path length of 12 mm). A sample of MIP-3α at a concentration of 0.5 mg/ml (65 μM) in 200 mM potassium phosphate buffer (pH 7.4) was equilibrated at 40,000, 50,000, and 55,000 rpm and 20 °C. Radial scans were taken every 2 h until equilibrium was reached. The average molecular mass was calculated using the program Ultracentrifuge II (38, 39).

**Chemotaxis Assay**—The migration of CCR6-transfected HEK293 cells was assessed with a 48-well microchemotaxis chamber technique as previously described (40). The incubation time was 5 h. The cells were suspended in, and all chemotactic proteins were diluted with, 20% isopropyl alcohol, 200 mM trisodium citrate, and 100 mM HEPES (pH 7.5). Hexagonal rods appeared rapidly, but continued to grow slowly for 1–2 weeks at 12 °C. For x-ray experiments, the crystals were frozen in liquid propane after washing in the same reservoir solution with the isopropl alcohol concentration raised to 40%. Prior to x-ray exposure, the crystals were stored in frozen propane popsicles under liquid nitrogen. The x-ray data were collected on a single crystal at beamline X9B (National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY) using an ADSC Quantum 4 CCD detector. CCD images were processed, merged, and scaled using DENZO and SCALEPACK (31). The data collection statistics are shown in Table I.

**Structure of Human MIP-3α/CCL20**

| Table I  | Data and refinement statistics |          |
|----------|--------------------------------|----------|
| Wavelength (Å) | 0.98 |          |
| Space group   | P6₁ |          |
| Unit cell (Å) | a = b = 70.82, c = 71.77 |          |
| Resolution range (Å)<sup>a</sup> | 20.0 to 1.70 (1.76 to 1.70) |          |
| R<sub>merge</sub><sup>a,b</sup> | 0.087 (0.620) |          |
| Total no. of observations | 99,101 |          |
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| No. of reflections | 19,365 |          |
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| Resolution range (Å) | 15.0 to 1.70 |          |
| R<sub>merge</sub><sup>c</sup> | 0.176/0.217 |          |
| No of all non-H atoms | 1231 |          |
| Water molecules | 169 |          |
| Heterogen atoms | 40 |          |
| Average B-factor (Å<sup>2</sup>) | 27.7 |          |
| Protein atoms | 26.5 |          |
| No. of disordered residues | 5 |          |
| r.m.s.d.<sup>e</sup> from ideality | 0.007 |          |
| Bonds (Å) | 0.034 |          |
| Angle distances (Å) | 0.028 |          |
| Plane distances (Å) | 0.028 |          |

<sup>a</sup> The highest resolution shell ranges are shown in parentheses.
<sup>b</sup> R<sub>merge</sub> = Σ[I(λ) - I(λ')]/Σ[I(λ)].
<sup>c</sup> R<sub>merge</sub> = Σ[I(λ)]/[Σ[I(λ')]].
<sup>d</sup> R<sub>merge</sub> = Σ[I(λ)]/[Σ[I(λ')]], where T represents a test set of reflexions (4% of the total, chosen at random, not used in the refinement).
<sup>e</sup> Root mean square deviation.
FIG. 2. Stereo drawing of the MIP-3α dimer. In each monomer, the peptide chain begins at Asp 2 and ends at Lys 65. This figure was generated using the program RIBBONS (74).

Chemotactic medium (RPMI 1640 medium containing 1% bovine serum albumin). A MIP-3α standard was obtained from PeproTech (Rocky Hill, NJ).

Antimicrobial Assays—Recombinant human MCP-1, hBD1, and hBD2 were expressed and refolded using standard methods (41). After purification, all three proteins gave single peaks upon reversed-phase chromatography and were assayed for proper folding and purity by crystallization trials. All three gave crystals under previously reported conditions (27, 28, 42). The assay for antibacterial activity is a slight modification of the standard colony-count method (43). Escherichia coli ATCC 25922 cells were grown to mid-logarithmic phase in Mueller-Hinton Broth II and then diluted to 1 × 10^8 colony-forming units/ml in 10 mM potassium phosphate (pH 7.4) containing 1% (v/v) trypsin soy broth. Cells (100 µl) were incubated in the presence of different concentrations of MIP-3α for 3 h at 37°C. The cells were then diluted serially in the same buffer, plated on Luria broth plates, and incubated for 18 h at 33°C; and the colonies were counted. The same method was used for Staphylococcus aureus ATCC 29213 and Candida albicans strains 99788 (amphotericin B-resistant) and 90028, except that the cells were grown to mid-logarithmic phase in tryptic soy broth. Bactericidal activity was expressed as the ratio of colonies on a control plate to the number of colonies counted on the sample plate. The LD_{50} is the concentration of protein that reduces the number of colonies by 90%.

RESULTS

Description of the Monomeric Structure—The topology of the human MIP-3α monomer is very similar to that of other related CC-type chemokines, with the characteristic antiparallel three-stranded β-sheet and C-terminal α-helix (Fig. 2). Ala 1–Phe 4 are disordered as judged by the absence of corresponding electron density; and Asp 5, the first residue modeled in our structure, is quite mobile (as reflected by high values of B-factors). Leu 15–Leu 15 form a meandering coil between the cluster of disulfide bonds and the first β-strand. A short β 10 helix located immediately prior to the first β-strand, consisting of Pro 17–Phe 19, is highly conserved in CC-, CXC-, and CX 3=C-type chemokine structures. Ile 20–Gln 26, Ala 36–Thr 41, and Ser 46–Ala 49 comprise the three β-strands (β1, β2, and β3, respectively) in the core β-sheet, with Leu 27–Asn 33 forming an extended β-turn and the Asn 35 oxygen hydrogen-bonding to the hydroxyl oxygen of Tyr 10. Thr 14–Leu 18 form the C-terminal α-helix. The C-terminal helix interacts with the β-sheet through the side chains of Leu 15, Ile 20, Phe 21, Ile 27, Phe 30, Trp 35, Val 36, Ile 56, Val 59, and Leu 61, creating a hydrophobic core spanning the secondary structure elements. Lack of interpretable electron density indicates that the five C-terminal residues, Lys 64–Met 69, are disordered.

The overall quality of the x-ray structure is very good. The average B-factors are 20.1 Å² for the backbone atoms and 33.1 Å² for the side chain atoms. Ramachandran plots (44), calculated using the program PROCHECK (45), show the conformations of all residues to be located within allowed regions. Although all the modeled residues are clearly defined by the experimental electron density, side chains of several residues (His 16, Thr 24, Arg 25, Leu 27, and Val 47) accommodate multiple conformations. The disulfides between Cys 6 and Cys 32 and between Cys 7 and Cys 48 display the canonical left-handed geometry as seen in most high-resolution chemokine structures.

The conformations of the two monomers present within the asymmetric unit are very similar, as reflected by a root mean square deviation (calculated for all atoms) of 0.55 Å. The dimer, formed by two crystallographically independent monomers, is of the α-type. In such a dimer, the β1-strands of both monomers interact through a series of hydrogen bonds and form a six-stranded antiparallel sheet, with both helices located on one side of the sheet (46). When both monomers are compared, however, it is evident that the side chains of two residues, Leu 15 and Trp 55, accommodate different conformations (Fig. 3). In monomer A, Trp 55 rotates away from the hydrophobic core between the C-terminal helix and the central β-sheet. This is quite unusual, as this tryptophan is structurally highly conserved in chemokines. However, its motion seems to be induced by both crystal packing and the presence of isopropyl alcohol. The side chain of this tryptophan residue is flanked by the side chains of Tyr 58 and Thr 54 from the same molecule, by Tyr 58 from a symmetry-related monomer, and by an isopropyl alcohol molecule. A conformational change of Trp 55 triggers the rotation of the Leu 15 side chain from the same molecule, placing it in the site left by the removal of the indole group of tryptophan. This motion is accompanied by a rotation of the Leu 15–His 16 backbone peptide. Such a rearrangement allows the indole nitrogen of Trp 55 to break a hydrogen bond with the side chain of Gln 53 from the same monomer and to form a new hydrogen bond with the carbonyl oxygen of Leu 15. The position of the backbone nitrogen atom in the rearranged peptide bond between Leu 15 and His 16 is additionally stabilized by the hydrogen bond with another isopropyl alcohol. Release of the tryptophan side chain from the core of the monomer is likely due to the presence of 40% isopropyl alcohol in the cryoprotectant solution; however, it indicates relative ease of such a rearrangement, previously not observed for chemokines.

Comparison of Human and Marine MIP-3α Structures—A comparison of the NMR structure of murine MIP-3α (26) with the crystal structure of human MIP-3α shows a number of differences (Fig. 4). Overall, the root mean square deviations for Co alignments between the ensemble of structures for murine MIP-3α and human MIP-3α ranges from 2.0 to 2.5 Å. This is higher than would be expected, as the mature amino acid sequence of human MIP-3α has 69% identity to that of murine MIP-3α (12). It is worthwhile to point out that root mean square deviation values obtained by equivalent comparisons of the structure of human MIP-3α presented here and the high-resolution structures of other chemokines (RANTES, MCP-1, and IL-8) are significantly lower (0.63, 0.73, and 1.46 Å, respectively). This observation is consistent with the fact that a successful molecular replacement solution could be found using a RANTES-based model in contrast to a murine MIP-3α-based model. The major differences between the structures of human
MIP-3α and murine MIP-3α lie in the loop following the disulfide cluster (Gly25–Phe19 in human MIP-3α) and the relative positions of the C-terminal helix and the core β-sheet. The cause of the shifts within the loop may be due to amino acid changes, as 8 of the 11 residues that are mutated between murine MIP-3α and human MIP-3α reside in this region. The shift is as much as 5 Å between equivalent Ca atoms. The C-terminal helix of human MIP-3α is less orthogonal to the core β-sheet, and its axis is straighter than that reported in murine MIP-3α. Besides the amino acid differences between the two sequences, the motion of the helix may also be associated with dimerization of the human protein. Although only the monomeric form of murine MIP-3α has been reported, murine MIP-3α may form similar dimers, as suggested by NMR information and ultracentrifugation studies (26).

There are also a number of minor differences between the human and murine MIP-3α structures. The disulfide bond between Cys8 and Cys25, reported in the structure of murine MIP-3α, accommodates a right-handed twist, opposite that seen in human MIP-3α. By comparison, the canonical left-handed twist is found in all high-resolution structures of CC-type chemokines reported to date. Although the main β-sheet is very similar, the turns between strands take different conformations. The conformations of the turn between the β1- and β2-strands (Asn21–Cys25 in human MIP-3α) are slightly different, with the main change in the position of Glu16. The turns between the β2- and β3-strands (Thr41–Leu45 in human MIP-3α) assume completely different conformations in both proteins. This is not unusual, however, as these fragments are highly mobile in both structures.

Analysis of Dimerization in the Crystal—The dimer formed by human MIP-3α monomers as seen in the crystal structure (Fig. 5) is similar to that seen for IL-8 (47). There are 10 hydrogen bonds between both monomers, four of which form between backbone atoms of two β1-strands. These interactions include the Val21 oxygen, Phe23 nitrogen, Phe23 oxygen, and Arg25 nitrogen atoms and lead to the formation of a symmetric antiparallel β-sheet spanning the dimer interface. Other hydrogen bonds are formed between the side chain of Arg25 and the carbonyl oxygen of Lys65, in addition to a very short hydrogen bond (2.3 Å) formed between Ser140 Oy atoms located within the helices of the two monomers. The symmetry of the dimeric interface is not exact, however, as only His40 Ne2 in one of two monomers forms a hydrogen bond with Thr24 Oy1 from the other monomer. Such asymmetry may be due to the presence of an isopropyl alcohol molecule that is located in the dimeric interface near the imidazole rings of His40 from both monomers.

A structural comparison of the MIP-3α dimer with other α-type chemokine dimers helps to evaluate the stability of the MIP-3α dimer in solution. Human SDF-1α has been shown to be monomeric in solution even at very high concentrations (48), whereas IL-8 forms very stable dimers at moderate concentrations (49). MCP-1 was also shown to form α-type dimers in a crystal, but it is not conclusive whether such dimers can also exist in solution (42). When calculated with GRASP (50), the solvent-accessible surface of MIP-3α buried upon dimerization is 1155 Å², or ~12% of the total surface. In comparison, the surface buried upon dimerization equals 1509 Å² for SDF-1α (16% of the total surface) (51), 1661 Å² for MCP-1 (16% of the total surface) (42), and 2276 Å² for IL-8 (23% of the total surface) (47). Concomitant with the amounts of buried surfaces, MCP-1 and SDF-1a form only four backbone hydrogen bonds across the dimer interface, whereas six hydrogen bonds are present in the case of IL-8. Thus, if MIP-3α forms dimers in solution, they likely are not very stable and present only at high protein concentrations.

The differences between the stability of α-dimers formed by MIP-3α and IL-8 can be explained after a structural alignment of both dimers (Fig. 5). It is seen that the β1-strands of both monomers of IL-8 are relatively straight and parallel until the turn into β2. In the case of MIP-3α, however, the corresponding strands are bow-shaped, with the closest distance to each other near the center of the dimer interface. Due to this topological change, two hydrogen bonds most distant from the center of the dimer are absent in MIP-3α. A similar bending of β1 can also be seen in SDF-1α and MCP-1. Two primary reasons can be deduced as determinants of β1 bending in MIP-3α. The first is a...
result of constraints placed on the β-turn (Asn^{29}–Cys^{32} in MIP-3α) by the formation of the Cys^{6}–Cys^{10} disulfide. The second arises from multiple hydrogen bonds between the side chain of Gln^{26} and the Cys^{7} nitrogen, Cys^{7} oxygen, and Ile^{34} oxygen backbone atoms. These interactions force the β-turn closer to the core β-sheet, buckling the Arg^{25}–Gln^{26} peptide and curling β1. The same constraints are also found for MCP-1, although the absence of a residue equivalent to Gln^{26} in MIP-3α within the loop region allows β1 to remain less bent than in MIP-3α. In IL-8, the presence of an additional residue between the first two cysteines (the CXC motif) allows the turn between β1 and β2 to be placed farther from the core β-sheet, thus removing strain on the geometry of β1 and allowing it to remain relatively straight. Although SDF-1α also has an additional residue between cysteines as in IL-8, an asparagine residue in β1, roughly equivalent to Gln^{26} in MIP-3α (Asn^{30} in SDF-1α), hydrogen-bonds across the dimer interface to backbone atoms in the other monomer, causing a buckling in β1 and disrupting dimerization.

Analytical Ultracentrifugation Studies—To further investigate the possibility of MIP-3α dimerization in solution, we conducted sedimentation equilibrium experiments at a pH and ionic strength similar to those used during crystallization, but at a significantly lower protein concentration (65 μM). The equilibria data collected for MIP-3α at pH 7.4 fit best to a model consisting of only one species with a molecular mass of 8 kDa, indicative of a monomer. Although the NMR measurements performed for murine MIP-3α indicated the formation of higher molecular mass aggregates (26), the corresponding experiments were conducted for protein concentrations of 1 mM, ~15 times higher than those used in this study. It is possible that human MIP-3α also dimerizes in solution at these concentrations, as evidenced by the crystal structure. Similar results were seen for other chemokines such as MCP-3 (52).

Chemotactic and Antibacterial Properties of Human MIP-3α—MIP-3α was subjected to a chemotaxis assay to determine its activity with CCR6-transfected HEK293 cells. Synthetic MIP-3α (both wild-type and truncated) showed maximum chemotactic activity at 1 and 10 ng/ml, similar to the MIP-3α standard used. The chemotactic profile for the synthetic MIP-3α was bimodal, although its maximum activity was higher than previously reported (13). The higher activity of the synthetic MIP-3α used in this study compared with the standard is likely due to the higher degree of sample purity. Both hBD1 and hBD2 show an ~10-fold lower activity than MIP-3α with the same cell type (24).

Given the similarities in chemotactic ability of both β-defensins and MIP-3α, it seemed plausible that MIP-3α might have antibacterial activity as well. MIP-3α was subjected to a time-based bactericidal assay (53) using E. coli ATCC 25922 along with hBD1, hBD2, and the CC-type chemokine MCP-1 as controls. MIP-3α showed an LD_{90} of 0.3 μg/ml; hBD1 showed an LD_{90} of 25 μg/ml; and hBD2 showed an LD_{90} of 6 μg/ml (Fig. 6A). Using the same assay, MCP-1 showed an LD_{90} of 30 μg/ml. The shorter form of MIP-3α (residues 1–65) showed indistinguishable activity under identical conditions. Although the activities of hBD1 and hBD2 are similar to those previously reported using the same assay (54–56), the relative activity of MIP-3α with respect to hBD1 and hBD2 (~20-fold more potent than hBD2) is surprising. The activity of MIP-3α was tested against Gram-positive S. aureus ATCC 29213 (Fig. 6B) and two strains of the fungus C. albicans (amphotericin B-sensitive and amphotericin B-resistant) using the same assay. MIP-3α showed an LD_{90} of ~60 μg/ml against S. aureus and showed no discernible antifungal activity against either strain of C. albicans. The LD_{90} values obtained for hBD2 using the same assay are >100 μg/ml against S. aureus and 25 μg/ml against C. albicans (55). Thus, although the antimicrobial activities of MIP-3α and hBD2 overlap, they differ in their optimal targets.

The physiological relevance of the bactericidal activity depends significantly on the in situ concentrations of MIP-3α. Although there is no definitive study stating the exact concentrations of MIP-3α present within epithelial tissues, it was shown that incubation of cultured primary human keratinocytes (80% confluence) with IL-1β and tumor necrosis factor-α induced MIP-3α to a level of 0.075 μg/ml in supernatants (6). Based on this level of induction, there would need to be between 2 × 10^{5} and 1 × 10^{6} cells/ml to reach antimicrobially effective concentrations of MIP-3α. These cell concentrations are certainly possible values for keratinocytes in epithelial layers, giving merit to the postulate that MIP-3α might serve as both a chemotactic and an antimicrobial agent.

Structural Comparison with β-Defensins—It has been shown that both hBD1 and hBD2 can chemoattract immature dendritic cells by binding to and activating CCR6 (24). Although there is little (if any) sequence homology between the β-defensins and MIP-3α, likely both β-defensins and MIP-3α share the structural motifs that allow them to compete for CCR6 binding. The high-resolution crystal structures of both hBD1 (27) and hBD2 (28) have been solved, and both display the same topology. The two β-defensins have a short helical segment at their N termini, followed by a small three-stranded β-sheet, all of which is cross-linked by three disulfide bonds. A cursory comparison of the structures of the two β-defensins and MIP-3α showed little similarity, aside from the obvious three-stranded β-sheet and proliferation of positively charged residues. A similar conclusion had been achieved in a comparison of murine MIP-3α and hBD2 (26).

**DISCUSSION**

Possible Determinants of the Highly Specific Interaction between MIP-3α and CCR6—A simple amino acid sequence comparison of MIP-3α and other chemokines provides only a small degree of insight into understanding its high receptor specificity. MIP-3α shares sequence and structural features observed for a number of other chemokines. Extensive structural com-
Fig. 6. Antimicrobial activity of MIP-3α. The assays were conducted according to the protocol described under “Experimental Procedures” against E. coli ATCC 25922 (A) and S. aureus ATCC 29213 (B). The percent of bacteria killed is the ratio of the total number of colonies estimated from control plates with no peptide added to the number of colonies counted on each plate. The LD₉₀ is the concentration of peptide that reduces the number of colonies by 90%.

Fig. 7. Cationic patches on MIP-3α, hBD1, and hBD2. The positively charged residues in MIP-3α, hBD1, and hBD2 are shown in this stereo drawing in blue, whereas the DCCL motif in MIP-3α along with the possible aspartic acid-hydrophobic residue pair in hBD1 and hBD2 are drawn in magenta. Additionally, the disulfide bonds are colored green. The superimposed monomers of hBD1 and hBD2 are shown on the left, and human MIP-3α on the right. MIP-3α residue numbers are shown in black; hBD1 residue numbers are shown in red; and hBD2 residue numbers are shown in blue.

Comparison of MIP-3α and other human chemokines shows that the receptor recognition motif (formed by the N-terminal loop and the turn between β2 and β3) is very similar in most chemokines compared. Apparently, the structural basis of the highly specific interaction between MIP-3α and CCR6 is rather subtle.

The structural elements of chemokines most critical to their interactions with receptors include the extreme N terminus preceding the first cysteine residue, the cysteine-flanking fragment (XCC, XCCX, XCCX, and XCCXXCX for C-, CC-, CXC-, and CXX(C-type chemokines, respectively), and a wide shallow groove formed by the loop preceding the β1-strand and the turn between the β2- and β3-strands (57–61). The groove region is important mainly for chemokine recognition and high-affinity binding. The extreme N terminus and cysteine-flanking sequence, although playing some role in recognition, are mainly important for receptor activation and signaling (62).

The interaction between chemokines and receptors can, in somewhat simplified form, be described as the combined interaction of those few motifs that are specifically presented to the receptor by the scaffold formed by the remaining fragments of chemokine molecules. An inspection of sequence and structural alignments uncovered several of these motifs in MIP-3α. One such motif is the cysteine-flanking fragment of MIP-3α, composed of the four residues Asp⁵-Cys⁶-Cys⁷-Leu⁸ (DCCL). As stated earlier, this motif should be pivotal for the receptor activation. The DCCL motif is also found in the sequences of MIP-3β/CCL19, Exodus-2/CCL21, and TECK/CCL25, whereas MIP-3/CCL23 and TARC/CCL17 consist of very similar sequences, DCCL and ECCL, respectively. Although these chemokines recognize different receptors, the mechanism of receptor activation most likely is the same. Extensive mutational analysis of various chemokines has shown that the residues flanking the first two cysteines are absolutely critical in chemokine-receptor interactions, as single residue mutants showed as much as a 100-fold decrease in binding affinity (57, 63–66).

Structural Features and Antimicrobial Properties of MIP-3α—In this report, we have shown, by means of antimicrobial assays, that MIP-3α has strong antibacterial properties. In our assays, MIP-3α was at least as potent as several of the β-defensins, proteins recognized primarily as natural antimicrobial agents. Although, to our knowledge, it is the first observation of MIP-3α having antimicrobial properties, similar activity has been previously reported for MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, CXC-type chemokines lacking an ELR motif (67). It was suggested that the antimicrobial activity was linked to an abundance of positive charge in the C-terminal helices of those chemokines. MIP-3α does indeed contain a high number of cationic residues in this region of the molecule. However, high content of positively charged residues in the C-terminal part is not unique to the mentioned chemokines and is indeed quite common across the whole family of chemokines. One such chemokine is MCP-1, also subjected to bactericidal assays, MIP-3α—having antimicrobial properties, similar activity has been previously reported for MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, CXC-type chemokines lacking an ELR motif (67). It was suggested that the antimicrobial activity was linked to an abundance of positive charge in the C-terminal helices of those chemokines. MIP-3α does indeed contain a high number of cationic residues in this region of the molecule. However, high content of positively charged residues in the C-terminal part is not unique to the mentioned chemokines and is indeed quite common across the whole family of chemokines. One such chemokine is MCP-1, also subjected to bactericidal assays in this study, which displays activity only at very high protein concentrations. Therefore, it is not the net charge, but rather its specific distribution that likely determines bactericidal potential. In comparison, defensins are characterized by a large number of cationic residues arranged around a rigid frame of three disulfide bonds, with only a few of these residues being conserved. In β-defensins, these conserved residues are located within a specific region of the molecule, between the C terminus and along β2.

Two such regions with high accumulation of cationic resi-
dyes, similar to that in β-defensins, can be found on MIP-3α (Fig. 7). One spans the turn between the N-terminal loop and the β-strand and the turn between the β2- and β3-strands (Lys18, Lys42, Lys43, and Lys49). The second flanks the C-terminal helix (Arg25, Lys52, Lys57, Arg61, Lys60, Lys66, and Lys68). The alignment of positive charges into distinct patches is very similar to that seen in hBD2. In is worth mentioning that the positively charged motifs in the C-terminal part of chemokines have been previously implicated in other roles such as glycosaminoglycan binding (68). Whether the proper distribution of positively charged residues is sufficient to render a protein bactericidal is not very clear.

Another aspect relevant to bactericidal activity is amphipathicity. This feature is crucial to the theory of pore formation (69, 70). Calculated electrostatic potential maps show only a small patch of hydrophobicity centered at Leu8, with points of negative charge surrounding this residue. Unlike human neutrophil peptide-3, a member of the α-defensins (71), very little of the surface of MIP-3α is hydrophobic. The lack of amphipathicity thus gives weight to the competing mechanism of bactericidal activity, molecular electroporation (72) or the carpet theory (73).

**Determinants of Chemotaxis: MIP-3α and β-Defensins**—A comparison of the entire structures of hBD1, hBD2, and MIP-3α does not reveal immediately extensive topological similarities. According to the earlier discussion, however, small structural motifs implicated in the chemotactic activity of MIP-3α should also be present in molecules of β-defensins. Of the three motifs described above, the first (consisting of the Cys2–Cys19 patch) can be observed in all β-defensins. Similar patch of hydrophobicity centered at Leu8, with points of negative charge surrounding this residue. Unlike human neutrophil peptide-3, a member of the α-defensins (71), very little of the surface of MIP-3α is hydrophobic. The lack of amphipathicity thus gives weight to the competing mechanism of bactericidal activity, molecular electroporation (72) or the carpet theory (73).

Identification of the recognition motif is less straightforward. Within an 8.5-Å radius of the conserved aspartate residues (Asp33 and Asp55), which are present in hBD1 and hBD2, respectively) are points of negative charge. This feature is crucial to the theory of pore formation (69), whereas the distance between the C-terminal helix (Arg25, Lys52, Lys57, Arg61, Lys65, Lys66, and Lys68) is probably due to their proximity to the first disulfide bond (Cys5–Cys12). Within an 8.5-Å radius of the conserved aspartate residues (Asp33 and Asp55), which are present in hBD1 and hBD2, respectively) are points of negative charge. This feature is crucial to the theory of pore formation (69), whereas the distance between the C-terminal helix (Arg25, Lys52, Lys57, Arg61, Lys65, Lys66, and Lys68) is probably due to their proximity to the first disulfide bond (Cys5–Cys12). Within an 8.5-Å radius of the conserved aspartate residues (Asp33 and Asp55), which are present in hBD1 and hBD2, respectively) are points of negative charge. This feature is crucial to the theory of pore formation (69), whereas the distance between the C-terminal helix (Arg25, Lys52, Lys57, Arg61, Lys65, Lys66, and Lys68) is probably due to their proximity to the first disulfide bond (Cys5–Cys12). Within an 8.5-Å radius of the conserved aspartate residues (Asp33 and Asp55), which are present in hBD1 and hBD2, respectively) are points of negative charge. This feature is crucial to the theory of pore formation (69), whereas the distance between the C-terminal helix (Arg25, Lys52, Lys57, Arg61, Lys65, Lys66, and Lys68) is probably due to their proximity to the first disulfide bond (Cys5–Cys12). Within an 8.5-Å radius of the conserved aspartate residues (Asp33 and Asp55), which are present in hBD1 and hBD2, respectively) are points of negative charge. This feature is crucial to the theory of pore formation (69).
Structure of Human MIP-3a/CCL20

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