Defensins are small (30–45 amino acid residues) cationic proteins with broad antimicrobial activity against many bacteria and fungi, some enveloped viruses, and other activities such as chemotaxation of a range of different cell types to the sites of inflammation. These proteins represent attractive targets for developing novel antimicrobial agents and modulators of immune responses with therapeutic applicability. In this report, we present the results of functional and structural studies of 26 single-site mutants of human β-defensin 1 (hBD1). All mutants were assayed for antimicrobial activity against Escherichia coli (ATCC strain 25922) and for chemotactic activity with CCR6-transfected HEK293 cells. To analyze the structural implications of mutagenesis and to verify the correctness of the disulfide connectivity, we used x-ray crystallography to conduct complete structural studies for 10 mutants in which the topology of disulfides was the same as in the native hBD1. Mutations did not induce significant changes of the tertiary structure, suggesting that the observed alterations of biological properties of the mutants were solely associated with changes in the respective side chains. We found that cationic residues located near the C terminus (Arg29, Lys31, Lys33, and Lys36) of hBD1 define most of the anti-E. coli in vitro activity of this protein. In turn, nearly all mutations altering the CCR6-mediated chemotaxis are located at one area of the protein, defined by the N-terminal α-helical region (Asp1...Ser8) and a few topologically adjacent residues (Lys22, Arg29, and Lys33). These experimental results allow for the first time drafting of the CCR6-epitope for a defensin molecule.

Defensins are recognized as an important element of the human innate immune system (1–4). The defensin family is composed of small (3–5 kDa), cationic, and cysteine-rich proteins. In human defensins, the connectivity of three disulfide bridges forms the basis for assigning them into one of two classes, the α- and β-defensins (5–7). In β-defensins, the topology of the three disulfide bridges is 1–5, 2–4, and 3–6, meaning that the first cysteine in the sequence is covalently linked to the fifth, and so on. Three human β-defensins, hBD1–3,4 have been isolated from natural sources and characterized in detail (8–11). Additionally, the recombinant or synthetic preparations of hBD4, hBD27, and hBD28 have also been described and characterized functionally (12, 13). Analysis of the human genome indicates the existence of over 40 potential coding regions for these peptides (14, 15).

In addition to broad antimicrobial activity, hBDs have also been recognized as modulators of cell-mediated adaptive immunity, due to their chemotactic and immunoenhancing activity (16–19). Recent studies revealed that β-defensins also play a role in cell differentiation, tissue remodeling, and sperm maturation (20–22).

The first human β-defensin to be discovered, hBD1, is constitutively expressed in the epithelial cells of the urinary and respiratory tracts and in keratinocytes of skin (9, 23, 24). A naturally occurring 36-amino acid hBD1 peptide shows antibacterial activity at micromolar concentrations against some Gram-negative bacteria (i.e. Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae), as well as yeast Candida albicans. When tested in vitro, hBD1 is relatively less potent against the Gram-positive Streptococcus aureus (9, 10, 23).

HBD1 and hBD2 selectively chemotactract human immature dendritic cells and memory T cells (17). The chemotactic activities of both defensins involve a specific seven transmembrane G12-protein-coupled receptor, the chemokine receptor CCR6 (17). The only known chemokine ligand of CCR6 is the macrophage inflammatory protein 3α (MIP-3α/CCL20), a constitutively expressed 9-kDa chemokine (17, 25–28).

A functional overlap between β-defensins and MIP-3α suggests that their biological activities may be determined by similar structural features (29, 30). Despite the lack of both amino acid sequence and structural similarity between β-defensins and MIP-3α, several laboratories suggested the molecular motifs that might be responsible for the common functions of these proteins (29–31). These motifs include a large, positively charged patch on the surface of the molecule and a cluster of residues in β-defensins that is topologically similar to the “Asp5-Cys6-Cys7-Leu8” fragment in MIP-3α. At the moment, however, these observations are purely hypothetical. Besides having a relatively
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Incomplete structure-function analysis of the antimicrobial properties of defensins, there is no experimentally derived base for similar analysis of their chemotactic properties.

To better understand the role of specific structural features in the chemotactic properties of \(\beta\)-defensins, we conducted extensive functional and structural studies of 26 single-site mutants of hBD1. All proteins were also subjected to anti-\(E. coli\) assays.

**EXPERIMENTAL PROCEDURES**

**Preparation, Expression, and Purification of the hBD1 Mutants**—The plasmids encoding the genes of the native hBD1 and all mutants were generated according to the protocol described elsewhere (32). The complete protocol for expression and purification of all proteins is also included in the supplemental materials. The molecular weights of folded and oxidized defensins were determined by electrospray ionization-mass spectrometry analysis. In the case of each protein, this analysis confirmed formation of three disulfide bonds. Subsequently, concentrations of defensins in appropriate buffers were determined by UV absorption spectrophotometry at 280 nm.

**Crystallization, Data Collection, and Processing**—Crystals were grown by the hanging-drop vapor diffusion method at room temperature with equal volumes of protein solution in water (usually at 20 mg/ml) and appropriate reservoir solution. The conditions promoting crystallization were determined by screening with the commercially available crystallization Sparse Matrix Screens. The final crystallization conditions are shown in the supplemental materials.

The X-ray diffraction data for most of the mutants were collected using a conventional radiation source. The intensities of reflections were recorded using a MAR Research 345 image plate detector mounted on a Rigaku RU-200 rotating-anode generator, operated at 50 kV and 100 mA. In the case of mutants L13A and K31E, diffraction data were collected using synchrotron radiation (beamline 22BM of the SER-CAT station at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL). The experimental intensities were recorded using the MAR CCD300 detector (MAR Research). In all cases, data reduction and scaling were performed with the program suite HKL2000 (33). The statistics obtained from the reduction of the x-ray data are shown in Table 1. All structures have been solved by the molecular replacement method using the program AMoRe (34), with the structure of the hBD1 monomer as a search model together, 17 residues representing 47% of the amino acid sequence of mature hBD1 were selected for substitution with Pro residues were excluded from mutagenesis. Also, neither of the mutated residues represent 60% of the solvent-accessible surface.

**Antimicrobial Activity Assay**—The antibacterial activity was determined using a slightly modified standard method (38) against \(E. coli\) cells (strain ATCC 25922). Cultures of \(E. coli\) were grown at 37 °C in tryptic soy broth to mid-log phase (\(A_{600}\) of 0.4 – 0.5) and then diluted to 10° colony-forming units/ml in sterile potassium phosphate buffer (KP, 10 mm, pH 7.4), supplemented with 1% (v/v) of tryptic soy broth. Bacteria were incubated for 3 h at 37 °C in the presence of different concentrations (between 0 (control) and 100 \(\mu g/ml\)) of hBD1 mutants. The \(E. coli\) cells were then diluted serially in the same buffer and spread onto LB agar plates (three for each concentration). After incubation for 24 h at 30 °C, the colonies were counted.

The anti-\(E. coli\) activity was calculated as a ratio of the number of colonies counted in the presence of defensin mutant to the number of colonies in the control plate. In each case, results were averaged over three independent experiments, and values of standard errors were determined. The final results are expressed as \(LD_{50}, LD_{90}\), and \(LD_{99}\), representing the concentrations of defensins causing death of 50, 90, and 99% of \(E. coli\) cells, respectively.

**Chemotactic Activity Assay**—A chemotaxis assay was performed, using a 48-well microchemotaxis chamber technique as described previously (39). CCR6-transfected human embryonic kidney cells (CCR6/HEK293) were suspended in RPMI 1640 medium containing 1% bovine serum albumin (pH 7.4) and applied at a density 5 × 10^5/ml. The same medium was used for dilution of the solutions of the mutants (at the concentrations 1, 10, 100, 1,000, and 10,000 ng/ml). During the assays, the CCR6/HEK293 cells were migrating through 10-μm filter membrane (Neuroprobe), pretreated (2 h, 37 °C) with collagen (g/ml rat collagen type I). After incubation at 37 °C for 5 h in humidified air with 5% CO₂, the filters were removed and the trapped CCR6/HEK293 cells were fixed to the membrane and stained using the HemaQuick kit (Richard Allen Scientific). The stained cells were then counted using the inverted microscope, under immersion oil. Each experiment was repeated at least five times, and the final results correspond to the averaged values with the appropriate standard errors. The results are represented by \(C_{\text{max}}\), indicating the concentration of peptide inducing maximum cell migration, and CI_{\text{max}} (the maximum chemotaxis index) defined as the -fold increase in the number of cell migration measured in the presence of test peptide over spontaneous cell migration in the presence of medium control.

**RESULTS**

**Selection of Point Mutations and Preparation of Defensin Peptides**—The residues subjected to mutagenesis were selected based on the earlier studies of the structural and physicochemical properties of human \(\beta\)-defensins (29, 35, 40 – 43). Altogether, 17 residues representing 47% of the amino acid sequence of mature hBD1 were selected for substitution with Ala and, in the case of several residues, also with other (usually charged) amino acids (Fig. 1). Additional criteria used during selection of residues subjected to mutagenesis were the surface accessibility and a predicted lack of the structural role. Based on the second condition, all Gly (with exception of Gly16), Cys, and Pro residues were excluded from mutagenesis. Also, neither of the two Ala residues was subjected to substitutions. The 17 mutated residues represent 60% of the solvent-accessible surface in hBD1(wt). An additional 12% of this surface is formed by the residues with well defined structural roles (Cys, Gly, and Pro) or alanines.

The wild-type hBD1 and the 26 mutants were expressed as recombinant insoluble fusion proteins in \(E. coli\). A straightforward protocol for isolation of peptides from the inclusion bodies and cleavage of the fusion leader allowed generation of milligram quantities of folded proteins that were purified to

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Combined with the analytical results, the phase-shift performance liquid chromatography, electrospray ionization mass spectrometry, and x-ray crystallography confirmed the purity, identity, and the correct fold of the proteins under study.

**Structural Analysis**—All hBD1 mutants included in this study were subjected to the crystallization trials to establish the extent of structural changes induced by mutations and to confirm the correctness of disulfide connectivity. For over half of the proteins, crystals formed readily during the routine screening procedure. However, crystals suitable for detailed crystallographic studies could be obtained for only 10 mutants (identified in Fig. 1). For each of the 10 mutants, the complete high quality x-ray data extended to at least 1.85 Å. These proteins crystallized under a variety of conditions, and, with few exceptions, their molecules were packed differently in the crystals (Table 1). In all cases, 35 of 36 residues in each molecule could be unambiguously modeled in the 2Fo−Fo electron density peaks, always including the site of mutation. Basic characteristics of the refined structures are shown in Table 1, whereas representative electron density maps and additional structural analyses are included in the supplemental materials.

The structural results for the 10 mutants of hBD1 provided clear answers to the two primary questions. None of the mutations induced significant changes of the tertiary structure. As shown in Fig. 2, the Cα traces of all crystallographically independent monomers representing the 10 mutants of hBD1 can be structurally well aligned with the six independent models of hBD1(wt), with the root mean square deviations between 0.18 and 0.66 Å. This level of topological similarity is comparable to root mean square deviations, 0.16–0.70 Å, calculated for the 45 possible alignments of 10 independent monomers of hBD1(wt) (see the supplemental materials). The main structural variability is observed for the loop connecting the strands β1 and β2 (the fragments Tyr14–Ala16 and Pro18–Thr21), which is inherently flexible in the hBD1 molecule. The largest difference in the conformation of this loop is found for the mutants L13A and K31E, where the shift of the Cα atom of Ile19 compared with the wild-type defensin is close to 2.8 Å. Smaller structural differences are also observed for some long surface-exposed side chains (i.e. Lys, Arg, and Glu). An analysis of B-factors (supplemental materials) shows that mutations did not significantly affect the dynamic properties of the proteins.

The crystallographic studies confirmed for all mutants the connectivity of six cysteine residues characteristic for β-defensins, proving the effectiveness of the applied folding protocol. The stereochemistry of three disulfide bridges in the mutated proteins is also identical to that determined earlier for hBD1 (35, 44, 45). Cumulatively, the structural results indicate that the differences in the biological in vitro activities of the hBD1 mutants result solely from the changes in the specific mutation sites, characterized either by the alterations to the geometry of molecular surface or to its physicochemical properties (such as electric charge, hydrophobicity, etc.).

The secondary result, obtained during these studies, is an observation that, despite the variability of crystal environments, none of these proteins exhibited well defined higher order arrangements (oligomerization, formation of the quaternary structure). This observation extends the earlier notions (35, 44, 45) that, in the absence of other molecules, hBD1 exists in the monomeric form. **Antibacterial Properties**—The antibacterial activities of the mutants and hBD1(wt) were tested against E. coli ATCC 25922 cells in a low ionic strength medium. Utilization of the uniform protocol allows for several significant observations. As a measure of anti-E. coli activity, we have chosen three different values of protein concentration, resulting in death of 50%, 90%, and 99% of bacteria. All experiments were performed at least three times. In parallel to the assay for each mutant, a similar experiment with the hBD1(wt) was conducted, allowing for the overall standardization of the results. The results of the experiments are shown in Table 2 and in the supplemental materials. Because the highest concentration of protein was 100 μg/ml, in cases when the particular E. coli lethality level was not achieved, the appropriate value of LD50 was not determined and is referred in the Table 2 as >100. In practical terms, the lack of satisfactory anti-E. coli activity of a protein at the concentration of 100 μg/ml justifies its classification as inactive or barely active.

Only one mutant, D1K, showed higher anti-E. coli activity than hBD1(wt). For 11 mutants (D1A, Y3A, S7A, G10A, Q11A, L13K, K22A, I23A, Q24A, Q24K, and T26A), the activity was comparable to the wild-type defensin. For the remaining 14 mutants, we observed a significant drop in the activity to a nearly complete disappearance in the case of five mutants (L13E, K22E, Q24E, K31E, and K33E). The critical role of basic residues for antibacterial properties of defensins is well established (8, 46–51). Consequently, mutations of those residues, especially when associated with charge reversal, result in a profound drop in the activity. However, not only charge reversal leads to the dramatic changes in anti-E. coli properties. Our results show that a similar effect may result from replacement of uncharged residues by an acidic residue (i.e. L13E and Q24E).
## TABLE 1
### Diffraction data and structure refinement statistics

| Mutant | N04A | S08A | Q11A | L13A | L13E | K22E | Q24A | Q24E | K31A | K31E |
|--------|------|------|------|------|------|------|------|------|------|------|
| PDB code | 2NLB | 2NLC | 2NLD | 2NLE | 2NFL | 2NLG | 2NLH | 2NLP | 2NLQ | 2NLS |
| Data processing | | | | | | | | | | |
| Wavelength (Å) | 1.54178 | 1.54178 | 1.54178 | 0.97180 | 1.54178 | 1.54178 | 1.54178 | 1.54178 | 0.97947 |
| Resolution range (Å) | 40-1.85 | 30-1.65 | 50-1.49 | 50-1.35 | 40-1.45 | 40-1.65 | 50-1.85 | 40-1.85 | 30-1.8 |
| Space group | P21 | P1 | P21 | C2 | P1 | P21 | C2 | C2 | P21 |
| Unit cell (Å) | a = 46.52, b = 26.40, c = 57.53, α = 90, β = 100.8, γ = 90 | a = 25.74, b = 33.19, c = 41.85, α = 90, β = 85.5, γ = 86.2 | a = 32.84, b = 23.57, c = 40.45, α = 90, β = 107.0, γ = 90 | a = 60.01, b = 22.78, c = 40.15, α = 90, β = 108.5, γ = 90 | a = 44.58, b = 46.79, c = 41.94, α = 90, β = 97.3, γ = 90 | a = 73.6, b = 59.64, c = 58.23, α = 90, β = 102.57, γ = 113.6, β = 113.5, γ = 90 | a = 90.0, b = 58.26, c = 75.12, α = 90.0, β = 97.9, γ = 90.0 | a = 18.81, b = 27.61, c = 59.64, α = 90.0, β = 97.9, γ = 90.0 | a = 18.81, b = 27.61, c = 59.64, α = 90.0, β = 97.9, γ = 90.0 |
| Measured | 62,153 | 94,174 | 46,585 | 18,657 | 62,576 | 26,732 | 18,657 | 62,576 | 100,477 |
| Unique | 12,053 | 14,783 | 9,213 | 8,495 | 13,851 | 14,844 | 13,851 | 14,844 | 16,163 |
| Redundancy | 5.2 (5.0) | 6.4 (5.0) | 5.1 (3.5) | 5.1 (3.5) | 2.2 (1.7) | 4.5 (3.6) | 2.2 (1.7) | 4.5 (3.6) | 2.2 (1.7) |
| Completeness (%) | 98.8 (95.1) | 92.6 (82.4) | 94.1 (77.2) | 96.6 (83.4) | 99.0 (97.2) | 92.6 (87.1) | 99.0 (97.2) | 92.6 (87.1) | 99.0 (97.2) |
| Rmerge <sup>a</sup> | 0.08 (0.26) | 0.05 (0.21) | 0.06 (0.27) | 0.04 (0.17) | 0.07 (0.19) | 0.04 (0.16) | 0.06 (0.51) | 0.06 (0.3) | 0.07 (0.39) |
| I/σ(I) <sup>b</sup> | 17.1 (5.9) | 19.0 (6.4) | 17.4 (4.5) | 18.4 (3.6) | 18.5 (5.8) | 16.2 (5.3) | 10.2 (2.8) | 15.6 (6.1) | 17.4 (2.0) |
| Structure refinement | | | | | | | | | |
| Resolution (Å) | 30-1.85 | 30-1.65 | 30-1.49 | 30-1.35 | 30-1.45 | 30-1.65 | 30-1.85 | 30-1.85 | 30-1.80 |
| Rcryst <sup>c</sup> | 0.179 | 0.166 | 0.134 | 0.151 | 0.140 | 0.163 | 0.188 | 0.179 | 0.183 |
| Rfree <sup>d</sup> | 0.238 | 0.204 | 0.199 | 0.198 | 0.184 | 0.195 | 0.243 | 0.237 | 0.256 |
| Number of non-H atoms | 1084 | 1092 | 538 | 541 | 574 | 1091 | 1076 | 1084 | 1068 |
| Protein | 223 | 214 | 158 | 230 | 261 | 261 | 197 | 278 | 269 |
| Water | | | | | | | | | 91 |
| Bond lengths (Å) | 0.016 | 0.019 | 0.17 | 0.018 | 0.018 | 0.017 | 0.019 | 0.018 | 0.018 |
| Bond angles (°) | 1.523 | 1.666 | 1.569 | 1.862 | 1.672 | 1.601 | 1.662 | 1.698 | 1.612 |
| Planarity (Å<sup>2</sup>) | 0.007 | 0.008 | 0.010 | 0.008 | 0.010 | 0.007 | 0.008 | 0.007 | 0.007 |
| Chiral centers | 0.094 | 0.119 | 0.147 | 0.113 | 0.113 | 0.108 | 0.104 | 0.104 | 0.177 |
| Average B-factor (Å<sup>2</sup>) | 21.6 | 17.3 | 14.3 | 15.5 | 13.5 | 20.8 | 31.7 | 23.9 | 23.5 |

<sup>a</sup> Values shown in parentheses correspond to the outermost resolution shell.

<sup>b</sup> R<sub>merge</sub> = Σ|I<sub>i</sub> - 〈I<sub>i</sub>〉|/ΣI<sub>i</sub>, where I<sub>i</sub> is the average intensity of symmetry-equivalent reflections.

<sup>c</sup> R<sub>cryst</sub> = Σ|Fo -Fc|/ΣFo.

<sup>d</sup> The value of free R<sub>factor</sub> was calculated based on a randomly chosen 5–10% of reflections excluded from refinement.
On the other hand, introduction of additional basic residues (L13K) or substitution of basic residues by an alanine (K22A) does not necessarily lead to noticeable effect on the anti-\textit{E. coli} activity. Observation of complete loss of the anti-\textit{E. coli} activity for mutants K22E and K31E, accompanied by virtually no structural changes induced by these mutations, confirms the role of positively charged, surface residues as a primary determinant of the anti-\textit{E. coli} properties of hBD1.

\textbf{Chemotactic Activity—}All mutants of hBD1 discussed here were subjected to the chemotaxis assays against the CCR6-transfected HEK293 cells. For standardization purposes, the assay for every mutant was paralleled by the same experiment with the wild-type hBD1 (the positive control) and in the absence of protein (the negative control). Additionally, in prior experiments with the hBD1 mutants, each batch of HEK293 cells was tested in the presence of 100 ng/ml CCL20 (MIP-3\textalpha) to assure a chemotactic responsiveness of the cells. The values of \(C_{\text{max}}\) indicating the concentration of defensin that induces maximum cell migration, and \(\%_{\text{max}}\) defined as the percentage of the chemotactic effect caused by hBD1(wt) at \(C_{\text{max}}\), concentrations of both the mutant and wild-type defensins, were calculated for each protein tested. Whereas \(C_{\text{max}}\) characterizes the binding of the ligand (defensin) to CCR6, \(\%_{\text{max}}\) reflects the ability of the ligand to activate the receptor. The average values of \(C_{\text{max}}\) and \(\%_{\text{max}}\) are shown in Table 3, the individual profiles of cell migrations at different concentrations of defensins are shown in Fig. 3, whereas the absolute cell counts are presented.

**TABLE 3**

| Mutation | Migration induced by hBD1 (wt) | Concentration inducing maximum cell migration, \(C_{\text{max}}\) |
|----------|--------------------------------|---------------------------------|
|          | %                              | \(\text{ng/ml}\)                |
| hBD1(wt) | 100 \(\pm\) 11                 | 100                             |
| D1A      | 87 \(\pm\) 14                  | 100                             |
| D1K\*    | 54 \(\pm\) 8                  | n/a                            |
| Y3A      | 94 \(\pm\) 11                  | 100                             |
| N44      | 40 \(\pm\) 8                   | 1000                            |
| V6A      | 143 \(\pm\) 13                 | 100                             |
| S7A      | 107 \(\pm\) 8                  | 1000                            |
| S8A      | 50 \(\pm\) 10                  | 1000                            |
| G10A     | 79 \(\pm\) 10                  | 100                             |
| Q11A     | 94 \(\pm\) 7                   | 1000                            |
| L13A     | 63 \(\pm\) 10                  | 1000                            |
| L13E     | 85 \(\pm\) 8                   | 1000                            |
| L13K     | 89 \(\pm\) 13                  | 1000                            |
| K22A     | 60 \(\pm\) 7                   | 100                             |
| K22E\*   | 34 \(\pm\) 7                  | n/a                            |
| I23A     | 90 \(\pm\) 11                  | 1000                            |
| V24A     | 81 \(\pm\) 10                  | 100                             |
| Q24E     | 130 \(\pm\) 8                  | 1000                            |
| Q24K     | 61 \(\pm\) 6                   | 100                             |
| T26A     | 97 \(\pm\) 7                   | 1000                            |
| R29A\*   | 57 \(\pm\) 8                   | n/a                            |
| K31A     | 109 \(\pm\) 14                 | 1000                            |
| K31E     | 98 \(\pm\) 8                   | 1000                            |
| K33A     | 104 \(\pm\) 10                 | 1000                            |
| K33E\*   | 29 \(\pm\) 7                   | n/a                            |
| K36A     | 79 \(\pm\) 11                  | 1000                            |

\* Typical (bell-shaped) profile (see Fig. 3) of the number of migrating cells versus defensin concentration was not observed.

\* n/a, not applicable.
in supplemental materials. HBD1 is a rather weak-to-modest chemotactic agent that induces only about a 2-fold increase in cell migration as compared with the medium. To increase the statistical significance of the results, for many mutants with deficient chemotactic properties in comparison to hBD1(wt), the experiments were repeated six and more times prior to averaging. Despite these efforts, for some mutants (D1K, K22E, R29A, and K33E) the chemotactic profiles lacked the characteristic bell shape (Fig. 3). For these mutants, evaluation of $C_{\text{max}}$ is not possible.

The summary of the results from studies of chemotactic properties of the hBD1 mutants is graphically shown in Fig. 4. It needs to be stressed that several very conserved and partially surface-exposed residues were not subject to mutagenesis. Those fragments of the molecule, however, may also interact with CCR6; yet, due to the topological conservation, they likely do not play a role differentiating affinity of various defensins for this receptor. Analysis of Fig. 4 shows that 13 residues determined here as involved in the interaction with CCR6 are distributed over most of the molecular surface of hBD1; however, it appears that contribution of residues located on one face of the monomer (left side of Fig. 4) is somewhat more extensive.

The N-terminal residues of hBD1 appear to contribute in a significant way to the CCR6 engagement. The N-terminal fragments of hBD1–3 (but not of $\alpha$-defensins) adopt an $\alpha$-helical conformation. Therefore, it seems plausible that this motif uniquely defines the affinity of human $\beta$-defensins for CCR6. The CCR6-epitope, extending over the molecular surface defined by the N-terminal fragment of hBD1, is enlarged by at least three other structurally adjacent residues (Lys$^{22}$, Gln$^{24}$, and Lys$^{36}$).

Some complication to the analysis stems from the fact that not all of the residues shown in Fig. 4 were subjected to the same type of substitutions (i.e. simple Ala scan), and different substi-
Antibacterial Activity—Several previous studies focused on the molecular determinants of antimicrobial activity in defensins (13, 40–43, 46–49, 51). The results consistently indicated the significance of both the basic residues and their asym-

tutions of the specific residue induced sometimes different effects. Only a single substitution to alanine was investigated for Tyr, Asn, Val, Ser, Gly, Glu, Gln, Ile, Thr, and Lys. Yet, even such modest replacements as V6A, S8A, or G10A resulted in significant changes of the affinity for CCR6. The results for the mutant V6A, the only one with significantly higher affinity for CCR6 than hBD1(wt), suggest a preference for a small residue in position 6 (a fragment of the N-terminal α-helix) of hBD1. In turn, a significant contribution to the CCR6 binding is likely provided by the hydroxyl group of Ser, whereas some flexibility (Gly) is preferred at or near position 10 of hBD1. For several residues (Asp, Lys, Ser, Gly, Lys, and Lys), the physicochemical properties rather than the size of their side chains are determinants for productive interaction with CCR6. In the case of these residues, mutations Lys → Glu or Asp → Lys resulted in much more pronounced effects than substitutions by alanines. Therefore, these residues more likely interact with the charged or polar fragments of CCR6. A different effect is observed for residue Arg. While the R29A mutant is practically inactive in binding and/or activation of CCR6, swapping charges in this position (R29E) does not affect activation of the receptor.

**DISCUSSION**

In this report, we present a first attempt toward the comprehensive and systematic search for the molecular/structural determinants of the chemotactic properties of human β-defensins. Previously, this aspect was addressed only hypothetically, based primarily on weak structural similarities between β-defensins and the chemokine, MIP-3α (29, 30). For this study, we prepared 26 single-site mutants of hBD1 in 17 different positions. Selection of mutants, based on the rationale described above, certainly does not include many significant possibilities. Although hBD1 is the poorest ligand of CCR6 among the human β-defensins characterized to date, it was used for this study, due to the ease of generating properly folded, soluble, and functional mutated derivatives. All the mutants were carefully characterized in terms of their purity, identity, and structural homogeneity, to minimize the ambiguity of interpretation. Although all proteins studied here were subjected to the bactericidal assay against a single strain of E. coli, providing additional information relating this activity with structural features of defensins, the main focus was directed toward the relationship between the structure and CCR6-mediated chemotactic activity of human β-defensins.

**Structural Studies**—All proteins included in this study were subjected to crystallization trials. However, only those proteins for which crystals suitable for diffraction experiments could be generated during the first round of automated screening (200 different conditions provided by commercial screens) were subjected to detailed structural studies. Complete high resolution x-ray structures were solved and refined for 10 such mutants. None of the studied proteins exhibited atypical disulfide connectivity or significant topological changes as compared with the wild-type hBD1. For each of the 26 hBD1 mutants, the folding process led to a single dominating specimen, characterized by nearly invariant mobility on a reverse-phase analytical column. Only one mutant, K36E, yielded similar amounts of several oxidation products; it was excluded from further studies.

Although detailed structural properties of hBD1 have been reported in the past (35, 44, 45), the 10 high resolution structures of the mutants represent six arrangements of molecules in the crystal and combined, provide information for 31 crystallographically independent monomers. In none of them did the mutation-altered (usually minor) structural changes extend beyond the mutation site. Thus, the structural framework of β-defensin molecules appears to tolerate a substantial variability of amino acid sequences and is also robust to the environmental changes (for instance, pH of crystallization conditions varies between 4.6 and 8.5). This observation is consistent with the existence of many proteins adopting a defensin-like fold despite rather low amino acid sequence homology (52). Consistent with previous results of studies in solution (44, 45), formation of distinct oligomers of hBD1 was not observed for any of the 10 structures. In all the mutants, the same few regions of their molecules are characterized by higher flexibility, expressed by the elevated values of B-factors and some conformational variability.
metric distribution over the molecular surface that led to amphiphilic properties of defensins. Some reports suggested the lack of relevance of structural characteristics of defensins for the antimicrobial properties of these proteins (13, 41, 46–48, 51, 52). Because the tertiary structure is invariant for the proteins described here, the results obtained do not address the significance of structure for activity of hBD1.

The set of 26 mutants of hBD1 allowed an extensive analysis of the contributions by specific residues to the anti-\textit{E. coli} activity. Because only one pathogen under low salt conditions was included in the assay, the conclusions cannot be easily generalized. Nevertheless, an invariant fold of all the tested mutants and the standardized experimental conditions strengthen the significance of the final results. The most significant determinant of the anti-\textit{E. coli} activity of hBD1 mutants is the net charge. The importance of basic residues for antimicrobial activity of defensins has been reported previously for bovine \textit{\beta}-defensin 2 and 12 (48, 51) and hBD3 (46, 47). A distribution of the residues important for anti-\textit{E. coli} properties of hBD1 over the molecule (surface) is illustrated in Fig. 5, which also shows the distribution of the electric charge on the surface of defensins. Table 4 illustrates the measures of anti-\textit{E. coli} activity (calculated based on the data in Table 2), with emphasis on the type of mutation and the net charge of the molecules of the mutants. For each X \rightarrow Y mutant, all proteins tested in this study can be classified into one of seven categories, depending on the electrostatic properties of residues X and Y (see the Remark column in Table 4). It is clear from Table 4 that the anti-\textit{E. coli} activity of the mutants increases with the content of basic residues. The “swapped charge” mutants have indeed the most altered anti-\textit{E. coli} activities, with K22E, R29E, K31E, and K33E being essentially inactive and D01K mutant being three to five times more potent than hBD1(wt). Such a simple correlation between the net positive charge and the anti-\textit{E. coli} activity was reported previously for the hBD3-derived peptides (46). This trend, however, was not observed for other pathogens (46). In this study, we show that, in the assays with \textit{E. coli}, correlation is also not quite straightforward. It could be expected that changes of

![Figure 5](jbc.org)
activity caused by basic (Lys and Arg) and acidic (Asp and Glu) would be opposite, yet the mutant D01A is not any more potent than hBD1(wt), whereas the mutants R29E, R29A, and K22A also appear to be more potent than expected from removing their positively charged side chains.

The charged residues are not the only ones to play a role in the antimicrobial properties of defensins (43, 47, 54, 55). In our anti-E. coli assays, mutants Y03A, N04A, V06A, S08A, and N04A are either inactive or very poorly active. Interestingly, these residues are located in the N-terminal fragment of the hBD1 molecule, with the first four residing within the α-helical region. Yet, mutations of a few other residues from this region (S07A, G10A, and Q11A) do not affect bactericidal properties of hBD1. Based on the studies of the CD spectra and the antimicrobial properties of hBD2 derivatives, Ancheva and coworkers (56) suggested that an Asp residue (equivalent to Asp1 in hBD1) may be important for the conformational stability of the helical motif in β-defensins, and as such it may contribute to the high antimicrobial activity of these proteins. Possibly, lack of a stable structure of the N-terminal fragment may be responsible for our failure to obtain diffraction-quality crystals of the D01A and D01K mutants of hBD1.

Chemotactic Activity—The observation that hBD1–3 use the same receptor (CCR-6) as the chemokine, MIP-3α, led to a suggestion that all these proteins share similar structural features and, possibly, the mechanism of receptor recognition and activation (29, 30). Careful comparison of the structures of human β-defensins with both human and murine MIP-3α indicated a single aspartate (Asp1 in hBD1), followed by the hydrophobic residue Tyr3 or Val16 (DHYNVCV motif in hBD-1), as a potentially important determinant of CCR6-mediated chemotactic activity of defensin. The DHYNCV fragment is equivalent to the DCCL motif in MIP-3α (29, 30), and in hBD1 it forms a well structured α-helix, restrained against the rest of the molecule by the first disulfide bond (35). Except for the report suggesting the role of β-defensin-specific network of disulfide bridges in CCR6-mediated chemotactic activity of defensins (41), there are no published studies of structural determinants of chemotaxis in defensins.

The results presented here indicate that a quite substantial fraction of the surface of a defensin molecule contributes to binding and activation of CCR6. This observation is consistent with an earlier report implying a significance of all extracellular domains of CCR6 for interactions with MIP-3α (28, 57, 58). The most significant finding, however, points toward the central role of the N-terminal (α-helical) region of the β-defensins molecules for signaling through CCR6. Indeed, nearly all residues from the N terminus of hBD1 (underlined here: DHY NC V S S) subjected to mutations caused a significant to dramatic decrease of the affinity for the receptor. His6, excluded from this study, possibly also plays a role in the interaction with CCR6. This result seems particularly interesting due to its agreement with a broader range of observations. The lack of CCR6-mediated chemotactic activity of α-defensins may be associated with the absence of the N-terminal residues preceding the first cysteine in these proteins. Also, the findings described here agree very well with predictions (the DHYNCV motif in HBD1) based on comparisons between β-defensins and MIP-3α. These predictions extend even beyond the N-terminal fragment of hBD1. For a few other residues (i.e. Arg29, Lys31, and Lys33), identified here as important for CCR6-mediated chemotaxis of hBD1, an equivalent can also be shown in

### Table 4

Relative anti-E. coli activity (RA) of hBD1 mutants as a function of the net charge and type of the mutation

| Protein | Net charge | Relative RA<sub>50</sub> | Relative RA<sub>90</sub> | Remark |
|---------|------------|-----------------|-----------------|--------|
| K22E    | +2         | <0.11 (0.55)<sup>a</sup> | <0.11 (0.11)    | Group I: positive → negative charge swap |
| R29E    | +2         | 1.28            | 1.28            |        |
| K31E    | +1         | <0.11           | <0.11           |        |
| K33E    | +1         | <0.11           | <0.11           |        |
| L13E    | +3         | <0.11 (<0.11)   | <0.11 (<0.11)   | Group II: insertion of negative charge |
| Q24E    | +2         | <0.11           | <0.11           |        |
| K22A    | +2         | 0.42 (0.42)     | 0.89 (0.26)     | Group III: removal of positive charge |
| R29A    | +1         | 0.8             | <0.11           |        |
| K31A    | +1         | 0.56            | <0.11           |        |
| K33A    | +1         | 0.18            | <0.11           |        |
| K36A    | +1         | 0.13            | <0.11           |        |
| WT      | +4         | 1 (0.61)        | 1 (0.58)        | Group IV: charge unchanged |
| Y03A    | 0          | 0.32            | <0.11           |        |
| N04A    | 0          | 0.13            | <0.11           |        |
| V06A    | 0          | 0.57            | <0.11           |        |
| S07A    | 0          | 0.62            | 0.84            |        |
| S08A    | 0          | 0.4             | <0.11           |        |
| G10A    | 0          | 0.6             | 0.92            |        |
| Q11A    | 0          | 1.21            | 1.04            |        |
| L13A    | 0          | 0.16            | <0.11           |        |
| I23A    | 0          | 0.55            | 0.9             |        |
| Q24A    | 0          | 0.63            | 0.79            |        |
| T26A    | 0          | 1.28            | 1.02            |        |
| D01A    | +5         | 0.98 (0.98)     | 0.92 (0.98)     | Group V: removal of negative charge |
| L13K    | 1.17 (1.2) | 1.5 (1.2)       |        | Group VI: insertion of positive charge |
| Q24K    | 0.55       | 0.91            |        |        |
| D01K    | +6         | 4.19 (4.19)     | 4.74 (4.74)     | Group VII: negative → positive charge swap |
| Averages |            | 3.63            | 0.69            | 0.63   |        |

<sup>a</sup> RA represents the fraction of the E. coli cell killing activity compared to the wild-type hBD1, i.e., for the mutant R29A, RA<sub>50</sub> = LD<sub>50</sub>(R29A)/LD<sub>50</sub>(hBD1(wt)).

<sup>b</sup> Values in parentheses represent the averages calculated over the proteins with a particular type of mutation described in the Remark column. Because the range of protein concentrations tested was limited to 100 μg/ml and the consequent lack of accurate data for LD values for the least active mutants, all the average values are overestimated. Due to this uncertainty, values of RA<sub>50</sub> and their averages are not included in this table. These data clearly indicate that, in addition to physicochemical properties of specific residues, their location within the defensin molecule plays a significant role, and the latter is determined by the shape (structure) of the molecule.
the MIP-3α molecule. Therefore, our results are the first experimental evidence suggesting a common or similar mode of interactions between CCR6 and both types of ligands.

Finally, it is important to point out that the presence of the N-terminal residues preceding the first cysteine in defensin molecules is not necessarily sufficient for their CCR6-mediated chemotactic activity. In three human defensins (hBD1–3) for which such activity was demonstrated, the N terminus accommodates an α-helical conformation. Requirement of the specific conformation for this activity, therefore, cannot be excluded. Additionally, studies of hBD1 with its rather weak chemotactic activity allow drafting only the first approximation of the CCR6-epitope on the surface of the defensin molecule. Further research will be necessary to improve the quality of present conclusions, primarily by extending studies on different human β-defensins and by using broader range of CCR6-expressing cells.

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