Unique Properties of Human β-Defensin 6 (hBD6) and Glycosaminoglycan Complex

SANDWICH-LIKE DIMERIZATION AND COMPETITION WITH THE CHEMOKINE RECEPTOR 2 (CCR2) BINDING SITE*

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**Background:** Defensins regulate leukocyte trafficking, and the extracellular matrix is known to be important for these functions.

**Results:** NMR studies of hBD6 indicate that glycosaminoglycan (GAG) induces dimerization and that the binding site overlaps with the CCR2-binding site.

**Conclusion:** GAG binding modulates multiple structural and dynamic properties of defensin.

**Significance:** This study provides novel structural insights and may help to understand how defensins orchestrate leukocyte recruitment.

Defensins are components of the innate immune system that promote the directional migration and activation of dendritic cells, thereby modulating the adaptive immune response. Because matrix glycosaminoglycan (GAG) is known to be important for these functions, we characterized the structural features of human β-defensin 6 (hBD6) and GAG interaction using a combination of structural and in silico analyses. Our results showed that GAG model compounds, a pentasaccharide (fondaparinux, FX) and an octasaccharide heparin derivative (dp8) bind to the α-helix and in the loops between the β2 and β3 strands, inducing the formation of a ternary complex with a 2:1 hBD6:FX stoichiometry. Competition experiments indicated an overlap of GAG and chemokine receptor CCR2 binding sites. An NMR-derived model of the ternary complex revealed that FX interacts with hBD6 along the dimerization interface, primarily contacting the α-helices and β2–β3 loops from each monomer. We further demonstrated that high-pressure NMR spectroscopy could capture an intermediate stage of hBD6-FX interaction, exhibiting features of a cooperative binding mechanism. Collectively, these data suggest a “sandwich-like” model in which two hBD6 molecules bind a single FX chain and provide novel structural insights into how defensin orchestrates leukocyte recruitment through GAG binding and G protein-coupled receptor activation. Despite the similarity to chemokines and hBD2, our data indicate different properties for the hBD6-GAG complex. This work adds significant information to the currently limited data available for the molecular structures and dynamics of defensin carbohydrate binding.

Human defensins have been described as a group of structurally diverse, multifunctional host proteins that are secreted in response to danger and are able to recruit and activate various leukocytes, particularly antigen-presenting cells, including dendritic cells. These proteins, also called alarmins, act as warning signals that alert the innate and adaptive immune systems (1, 2).

The structure and function of defensins are very similar to those of chemokines, a superfamily of some 50 (8- to 12-kDa) proteins that are involved in leukocyte trafficking and activation (3). It has been demonstrated previously that human β-defensins (hBDs) 1–3 bind to the chemokine receptors CCR2 and CCR6 and induce chemotaxis (4–7).

There is now substantial evidence that chemokines regulate every step of the recruitment process via interactions with the cell surface and free glycosaminoglycans (GAGs)3 on endothelial cells and extracellular matrices. It has been thought that, within tissues, concentration gradients of chemokines are formed and maintained to establish directional signals for migrating cells, and this process is believed to be controlled by intermolecular complexes with GAGs (8, 9).

GAGs such as heparin and heparan sulfate (HS) are long, linear, sulfated polysaccharides. Heparin, which resembles certain sequences found in HS, is used widely therapeutically and is readily available in large quantities. Therefore, it is often used as a model for the investigation of GAG interactions. Heparin and HS are composed of disaccharide-repeating units of alternating glucosamine and uronic acid. The diversity stems from

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3 The abbreviations used are: GAG, glycosaminoglycan; HS, heparan sulfate; FX, fondaparinux; ITC, isothermal titration calorimetry; CSP, chemical shift perturbation; HSQC, heteronuclear single quantum coherence.
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...molecular level, all characterized chemokines are monomers, dimers, and also higher-order structures in solution (16, 17). Upon GAG interaction, chemokines oligomerize, enhancing their effects on high-affinity receptors within the local microenvironment (18, 19). Experiments have confirmed the in vivo biological significance of chemokine oligomerization. Chemokines CCL2, CCL4, and CCL5 mutated at the GAG binding site retain chemotactic activity in vitro but are unable to recruit cells when administered intraperitoneally. These data demonstrate that both GAG binding and the ability to form oligomers are essential for the activity of particular chemokines in vivo (19–21).

However, the role played by the defensins in GAG recognition is still poorly understood. The binding sites for GAGs on hBD2 and the dimeric state of the hBD2-GAG complex were described recently using NMR spectroscopy and mass spectrometry (22). To further examine this interaction, we chose to study hBD6, a monomeric defensin that is constitutively expressed in epithelial cells from the epididymis, testis, and lung (23) and known to bind to the N-terminal sulfopeptide of the CCR2 receptor (24).

Here we investigate the structural features of the defensin-GAG complex using two GAG models: the heparin pentasaccharide (fondaparinux, FX) and an octasaccharide heparin derivative (dp8). We mapped the interaction of hBD6 with FX and characterized their binding using a combination of NMR spectroscopy, high pressure, relaxation parameters, computational analysis, and isothermal titration calorimetry (ITC)-based methods.

Our results reveal that FX binds along the N-terminal helices and to the loops between the β2 and β3 strands of hBD6, promoting the formation of a ternary complex. Additionally, binding studies with a CCR2 N-terminal sulfopeptide demonstrate overlap and competition with the FX binding interface. This NMR study describes the structural and dynamic characterization of hBD6-GAG recognition, which, we suggest, may be involved in the regulation of defensin signaling.

EXPERIMENTAL PROCEDURES

Sample Preparation—15N-Labeled hBD6 was expressed and purified as described previously (24). NMR samples for the titration experiments and high pressure contained 0.1 mM 15N-labeled protein in 10 mM sodium phosphate buffer, 0.02% NaN3, and 90/10% (v/v) H2O/D2O (pH 5.0). Fondaparinux was purchased from GlaxoSmithKline. The heparin-derived octasaccharide (degree of polymerization, dp8) was prepared by size exclusion chromatography (SEC) of a commercially available enoxaparin sample applied to a Bio-gel P10 column as described elsewhere (25).

NMR Experiments—NMR experiments were performed on a Bruker DRX 600 equipped with a 1H,15N,13C TXI cryoprobe or Bruker Avance III 800-MHz instruments at 25 °C. hBD6 HN (amide hydrogens and nitrogens) assignments were transferred from chemical shift tables published previously (Biological Magnetic Resonance Bank code 18634). In titration experiments, heparin-derived oligosaccharides (3.7 mM) were added progressively to an hBD6 sample (0.1 mM) in the abovementioned buffer. For titration of FX into hBD6, the following molar ratios of GAG to defensin were used: 0:1.1, 0.2:1, 0.3:1, 0.5:1, 1:1, 2:1, 3:1, and 4:1. Titrations of dp8 into hBD6 were carried out with the following ratios: 0.25:1, 0.5:1, and 1:1. The pH value at each step of the titration was kept constant. Chemical shift perturbations were calculated using CcpNmr analysis (26). Dose-dependent changes in hBD6 CSPs upon titration with FX were fit using a non-linear equation. The dissociation constant (Kd) for the hBD6-FX complex was calculated as described previously (24).

The synthetic sulfopeptide (purity >95%) corresponding to the N-terminal of chemokine receptor CCR2 was purchased from American Peptide. The peptide has the following sequence: CCR2(sulfated)-15N-EVFTFFDYPDYGAP31. Binding site competition between the CCR2 sulfopeptide and FX was assessed using 1H,15N HSQC experiments at 25 °C. Titrations of 1 mM FX into a mixture of 400 μM CCR2 sulfopeptide, 100 μM 15N-labeled hBD6, 10 mM phosphate buffer (pH 5.0), and 10% (v/v) D2O were monitored by measuring the chemical shift changes in two-dimensional 1H,15N HSQC.

15N Backbone Dynamics—Relaxation experiments were recorded on 100 μM 15N-labeled hBD6 at a field of 800 MHz. 15N R1 and R2 relaxation rates were measured from spectra with different relaxation delays: 20, 50, 100 (duplicate), 200, 250, 500, 750, and 1000 ms for R1, and 48, 80 (duplicate), 112, 144, 176, 208, 240, 272, and 304 ms for R2. The errors in the peak intensities were calculated from the duplicate experiments. Relaxation data curve fitting was performed using CcpNmr analysis. Rotational diffusion tensor fitting and analysis of internal mobility were performed using the Tensor2 program (27).

Analytical Size Exclusion Chromatography—Size exclusion chromatography was carried out using a Superdex peptide 10/300 GL column (GE Healthcare) attached to an AKTA prime HPLC system, with detection conducted at 214 nm. The samples were run at 0.5 ml/min in 20 mM sodium phosphate (pH 5.0 and 7.4).

Titration Microcalorimetry Measurements—ITC experiments were performed at 25 °C with an iTC200 microcalorimeter (GE Healthcare). hBD6 and fondaparinux were dissolved in 20 mM sodium phosphate buffer, 50 mM NaCl (pH 5.0). The sample cell was filled with 100 μM hBD6 and the injection syringe with 1500 μM FX. The titration typically consisted of a preliminary injection followed by ~15 subsequent injections with 150-s spacing. The heat of dilution of the FX and the heat of dilution of the buffer were small compared with the heat of binding and were subtracted from the experimental titration results. The experimental data were integrated and fitted to a theoretical single binding site titration curve using Origin 7 software.
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RESULTS AND DISCUSSION

NMR Mapping of hBD6 Residues Altered by FX—We used NMR spectroscopy to map the direct interaction between hBD6 and GAG and to obtain high-resolution mapping of the binding interface of their complexes. The observed NMR chemical shift changes may result from direct interaction with the binding partner or from binding-induced conformational changes. We used the synthetic heparin pentasaccharide (48) FX as a highly sulfated HS mimetic and monitored its binding to hBD6 using two-dimensional $^1$H,$^1^5$N HSQC NMR spectroscopy. The residues that exhibited large changes in chemical shift were correlated with significant involvement in FX binding. The $^1$H,$^1^5$N HSQC spectrum of free $^1^5$N-labeled hBD6 assigned previously (24) was used to monitor chemical shift changes of amide resonances upon binding to FX.

Fig. 1A shows a detailed region of the $^1^H$,$^1^5$N HSQC NMR spectra for hBD6 recorded as a function of the FX concentration, ranging from a 0 (black) to a 4:1 FX:hBD6 ratio (cyan). Moreover, NMR titration experiments revealed large changes in hBD6 chemical shift in fast exchange equilibrium between free and FX-bound hBD6, as demonstrated by a gradual shift in peak positions in $^1^H$,$^1^5$N HSQC. We did not observe any changes such as precipitation or signal disappearance upon FX addition.

A quantitative analysis of the $^1^H$ and $^1^5$N chemical shift changes for a subset of hBD6 resonances following titration with FX using a simple binding model yielded a $K_d$ of 4.1 ($\pm$ 2.9) $\mu$M (Fig. 1B). Interestingly, both hBD2 and chemokines can interact with a heparin mimetic with similar micromolar affinities (22, 32). Fig. 1C shows the CSP map of hBD6 upon complete saturation (FX:hBD6 molar ratio of 4:1) plotted as a function of the residue number of hBD6 for the FX titration. It is clear that the hBD6 residues most sensitive to FX binding are located in the $\alpha$-helix (Phe-1, Phe-2, Asp-3, Glu-4, Lys-5, Cys-6, and Asn-7) and the loops between the $\beta$2 and $\beta$3 strands (Cys-27, Gln-28, Lys-29, Ser-30, Leu-31, and Lys-32). Three of seven lysines (Lys-5, Lys-29, and Lys-32) had backbone NH ($^1^5$N and $^1^H$) resonances affected significantly by the binding of FX (Fig. 1). The perturbed residues indicate that Coulombic interactions contribute to FX binding. Fig. 1D shows the FX binding site mapped onto the hBD6 structure (PDB code 2LWL).

Our data indicate that Lys-5 is located near Lys-32. This result suggests that, together, Lys-5, Lys-29, and Lys-32 make up the common heparin-binding site BBX6 motif, which is usually observed in the heparin-binding proteins. B and X stand for basic and neutral amino acids, respectively (12).

Isothermal Titration Calorimetry for Complex Characterization—The binding of hBD6 to FX was investigated using ITC to obtain a clearer picture of the binding stoichiometry and binding mechanism. The raw ITC data showed that the binding of FX to hBD6 is exothermic, resulting in negative peaks in the plots of power versus time. The integrated heat values were fitted with a one-site binding model to obtain a binding stoichiometry of $n = 0.5 \pm 0.03$ and an equilibrium $K_d$ of 10.4 $\pm$ 27 $\mu$M. The stoichiometry and equilibrium dissociation constant are both in agreement with the results derived from the
NMR data and are consistent with a 2:1 hBD6:FX stoichiometry (Fig. 2).

Mapping hBD6 Interaction with a Heparin Octasaccharide (dp8)—We also monitored the binding of a heparin-derived octasaccharide (dp8) using two-dimensional $^1$H,$^{15}$N HSQC NMR experiments. Residues that exhibited the largest changes in CSP correlated with the residues with large CSP changes observed for FX (Fig. 3). Saturation of the binding site was reached at a 2:1 hBD6:dp8 ratio, after which a further chemical shift change was negligible for all residues. CSP showed major chemical shift changes for the NH signals of residues Phe-1, Phe-2, Asp-3, Glu-4, Asn-7, Cys-27, Gln-28, Lys-29, Ser-30, and Leu-31.

Binding to GAG Promotes hBD6 Dimerization—We used backbone dynamics to further characterize hBD6-FX recognition. We examined the picosecond-nanosecond and microsecond-millisecond time scale conformational dynamics of the free hBD6 and the FX-bound form using $^{15}$N relaxation parameter measurements.

Fig. 4 shows the $^{15}$N $R_1$ and $R_2$ relaxation rates and $R_2/R_1$ values for hBD6 and the hBD6-FX complex at a 1:1 molar ratio. Remarkably, both $R_2$ and $R_2/R_1$ showed that the region exhibiting extensive microsecond-millisecond conformational exchange, in both the free and bound states of hBD6, encompasses part of the binding site for GAG formed by residues from the $\alpha$-helix. Interestingly, residues Lys-29, Ser-30, and Lys-32 showed increased $R_2$ values in the presence of FX when compared with hBD6 free in solution, indicating that the binding interface becomes dynamic at a microsecond-millisecond time scale in the hBD6-GAG complex (Fig. 4B and C). These data demonstrate that Ser-30, together with Lys-5, Lys-29, and Lys-32, is likely to contribute to the development of the BB$X_B$ motif observed in heparin-binding proteins. Another interesting feature of this defensin is that the picosecond-nanosecond dynamics observed in the first residues located in the $\alpha$-helix in the free state were reduced in the FX-bound form, indicating that the $\alpha$-helix becomes more structured upon FX binding (Fig. 4, B and C).
The formation of the hBD6-FX complex was confirmed independently by the overall decrease in $^{15}$N $R_1$ associated with an increase in $^{15}$N $R_2$, indicating an increase in the overall tumbling time of the protein. The rotational correlation time ($\tau_c$) for the complex was 7.4 ns, whereas that for the free protein was 3.4 ns, suggesting the formation of a ternary complex. The $^{15}$N $R_2$ relaxation rates recorded at a 2:1 molar ratio for both hBD6-FX and hBD6-dp8 were similar (data not shown).

The rotational correlation time demonstrates that the hBD6-FX ternary complex consists of two hBD6 molecules cooperatively bound to one FX. It is worth noting that the C terminus of hBD6 remains flexible in the bound state, indicating that the amino acids located in this region are not involved in FX binding.

The hBD6 dimerization/oligomerization state was probed by size exclusion chromatography studies at pH 5.0 and 7.4 (Fig. 4D). The hBD6-FX complex eluted in a position corresponding to a dimeric state on the basis of comparison with a standard protein, cytochrome c (12 kDa). As expected, the free hBD6 sample eluted in a monomeric state (24). These results, combined with the relaxation parameters, indicate that the GAG bound to hBD6 selectively induces the formation of dimers rather than higher-order oligomers.

**Modeling of the hBD6/FX Ternary Complex**—The hBD6-FX ternary complex model was obtained by data-driven docking. The NMR data were used as input for the multibody docking protocol on the HADDOCK web server. The resulting 400 structures after water refinement were grouped into eight clusters (using a cutoff of 5.0 Å), with the largest cluster also exhibiting the lowest HADDOCK score. The lowest energy structure of the NMR-restrained hBD6-FX ternary complex is displayed in Fig. 5 in both ribbon and surface charge forms. The structure shows that FX binds along the N-terminal helices proximal to the loops between the $\beta 2$ and $\beta 3$ strands (Fig. 5A).

As a consequence of its heparin structure (33, 34), the negative charges on heparin are distributed on both faces and can be presented to proteins on either side. An analysis of the structure shows that the number of contacts is not equal in both monomers. Three basic charge residues from each monomer, Lys-5, Lys-29, and Lys-32, participate as a possible heparin-binding BBXB motif through electrostatic interactions with the negatively charged sulfate groups of FX (Fig. 5B). Lys-5 from both monomers interacts with the sulfate groups of FX. Lys-29 of monomer A and Lys-32 of monomer B form electrostatic interactions with the sulfate groups of FX. Residues Asp-3, Ser-30, and Leu-31 of the hBD6 monomer B are involved in additional interactions with FX. In addition to the hydrogen bonds mediated by the sulfate moieties, the hBD6-FX ternary complex is also stabilized by protein-protein contact, specifically by hydro-
phobic interactions between the aromatic rings of Phe-1 residues from each monomer (Fig. 5D). Our interaction model explains the decrease in picosecond-nanosecond dynamics for the Phe-1 residue, indicating that the $\alpha$-helices in the ternary complex become more structured upon FX binding (Fig. 4).

Analyses of the electrostatics properties of this ternary complex model indicate that the helices and the loops between the $\beta_2$-$\beta_3$ strands induce a strong symmetry in charge distribution that is characterized by a very large positive electrostatic potential in the binding site (Fig. 5C).

These results, together with NMR experiments, suggest a “sandwich-like” model in which two hBD6 molecules bind a single FX chain. In this model, hBD6 molecules anchor sulfate groups from opposite sides to produce a dimer.

Despite the similarity to chemokines, our data are different from previous structural models (32, 35–37). Where the oligosaccharides bind at the surfaces of the chemokine dimer interfaces, we found that hBD6 can bind to FX, resulting in a sandwich-like dimer. Our results clearly indicate a unique property for the hBD6-FX complex. A similar mechanism was proposed for the recognition of chitin oligosaccharides by the chitin elicitor binding protein (38).

High-pressure NMR Studies of Free hBD6 and the hBD6-FX Ternary Complex—To confirm the sandwich-like dimerization, we investigated the effect of high pressure on the structure and dynamic properties of hBD6 in the free state as well as in the presence of FX.

Oligomeric proteins are known to be particularly sensitive to pressure, and several reports have clearly shown that pressure causes oligomers to dissociate into monomers (39–41). Specifically, we tested whether we could alter the population or manipulate the association/dissociation equilibrium of the ternary complex in an attempt to identify the hBD6 residues directly involved in FX binding.

An overlay of the 2D $^1$H,$^15$N HSQC spectra recorded for the free $^{15}$N hBD6 sample and in the presence of FX at 1–2400 bar...
FIGURE 5. Structural model for the hBD6-FX ternary complex. A, the lowest energy structure of the hBD6-FX complex within the highest-score NMR-restrained HADDOCK. Residues show substantial CSPs upon FX titration in cyan (α-helix) and magenta (β2-β3). Fondaparinux is depicted as a stick representation. B, details of interactions of FX with hBD6. The main interaction occurs through the side chains of lysines (blue). Hydrogen bonds and salt bridges formed with the sulfate moieties (obtained by HADDOCK output) are shown as yellow dashed lines. C, electrostatic potential was generated with the PyMOL program (Delano Scientific LLC). The surface polarity is scaled with colors, using blue for the positively charged surface and red for the negatively charged surface, whereas white indicates nonpolar patches. D, hydrophobic interactions between the aromatic rings of Phe-1 residues from each monomer of hBD6 are highlighted by dots.

FIGURE 6. Structural information for the hBD6-FX bound state under high-pressure NMR. Shown is an overlay of $^1$H,$^15$N HSQC spectra of free hBD6 (A) and the hBD6-FX complex (B) recorded at different pressures: 1 bar (pink), 500 bar (dark blue), 1000 bar (light blue), 1700 bar (cyan), and 2400 bar (green). C and D, plot of the first order ($B_1$) and second order ($B_2$) coefficients of the pressure-induced $^1$H chemical shifts for individual amide groups. Black circles, free hBD6; green circles, hBD6-FX complex. E, $^1$H,$^15$N CSP between the free hBD6 at 2400 bar and hBD6-FX complex at 2400 bar.
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is shown in Fig. 6, A and B, respectively. The hBD6 resonances changed with pressure and did not unfold at pressures up to 2400 bar, with signals remaining well dispersed under both conditions. All peaks shifted with pressure in a fully reversible manner under both conditions, indicating that the structural changes that occurred are fully reversible. Additionally, the hBD6 resonances showed large 
$^{1}\text{H}$, 
$^{15}\text{N}$ downfield shifts for all amide cross-peaks, with the notable exception of Glu-4, Asn-7, Lys-10, Gly-11, Gly-18, Glu-22, Cys-27, and Arg-35, which showed upfield 
$^{1}\text{H}$, 
$^{15}\text{N}$ shifts with increasing pressure.

The amide 
$^{1}\text{H}$ and 
$^{15}\text{N}$ chemical shifts were traced individually and indicated a distinct non-linearity with pressure in many of the peaks of hBD6 in the free and complex forms (data not shown). The linear and non-linear coefficients 
$B_1$ and 
$B_2$ for individual amides were determined on the basis of the equation mentioned under “Experimental Procedures”

$$ \text{CSP} = B_1 \times \text{pressure} + B_2 \times \text{pressure}^2 $$

We found that the values of 
$B_1$ are almost equal for the free and complex forms (Fig. 6C). The non-linear coefficients 
$B_2$ also showed similar values for most of the residues, but we found significantly larger values in the complex than in the free form, specifically for residues Phe-2, Asp-3, and Lys-8 located in the 
$\alpha$-helix and Glu-21 located in the 
$\beta$2-
$\beta$3 loop (Fig. 6D). By inspection of the 
$B_2$ data, one can recognize that the 
$\alpha$-helix is strongly involved in structural transitions.

The 
$^{1}\text{H}$, 
$^{15}\text{N}$ CSP between the free hBD6 at 2400 bar and hBD6 bound to FX at 2400 bar showed that the larger changes were at residues located in the 
$\alpha$-helix and in the loops between the 
$\beta$2 and 
$\beta$3 strands (Fig. 6E). These data indicate that, even at high pressure, FX remains bound to hBD6.

The plots in 
$D$ and 
$E$ were obtained with the Tensor2 program. The overall rotational correlation times were 3.8 ns at 2400 bar free in solution and 4.6 ns at 2400 bar in complex with FX.

Interestingly, the overall correlation time for the hBD6-FX complex under pressure decreased from 7.4 to 4.6 ns. Free hBD6 exhibits a 
$\tau_r$ value of 3.8 ns at 2400 bar and 3.4 ns at 1 atmosphere. Therefore, the 
$\tau_r$ of hBD6-FX at 2400 bar is only 1.2-fold larger than that of free hBD6 (3.8 ns). This is in good agreement with the partial ternary complex dissociation and indicated that the application of high pressure (2400 bar) to the hBD6-FX ternary complex induced the dissociation of one hBD6 molecule, resulting in a binary complex formed by one hBD6 molecule and one FX molecule. These results clearly confirm that two hBD6 molecules simultaneously bind to one FX molecule from opposite sides, resulting in the dimerization of hBD6 by a sandwich-like dimerization mechanism. Additionally, combining the CSP and 
$\tau_r$ results, we can conclude that the contribution of the dimerization interface on the CSP data is small and that it is dominated by the contribution of GAG binding.

FX and CCR2 Sulfopeptide Compete for hBD6 Binding—We demonstrated recently that hBD6 interacts with a peptide corresponding to the extracellular domain of CCR2 containing sulfation on Tyr-26 and Tyr-28 (24). The heparin-binding surface identified in this study by chemical shift mapping is shared with the CCR2 sulfopeptide surface. The analysis of the chemical shift changes suggests that nearly the same residues in hBD6 are sensitive to the CCR2 sulfopeptide (Fig. 8A, red) and FX binding (Fig. 8A, blue).

Similar to the chemokine system, defensins are presumed to require GAG in the extracellular matrix to be presented to the receptor, transmitting the external migratory signal to the internal cellular machinery. The initial interaction with chemokine receptors is mediated through the N terminus of the receptor (44–46).
Because of the overlap of hBD6 binding sites for the CCR2 sulfopeptide and FX, it is reasonable to assume that their binding to hBD6 is competitive. To evaluate this possibility, we performed NMR binding competition experiments. The competition between the CCR2 sulfopeptide and FX for hBD6 binding sites can be illustrated by NMR titration experiments performed for $^{15}$N-labeled hBD6. Fig. 8A shows the superposition of the $^1$H,$^{15}$N HSQC NMR spectra of hBD6 upon addition of the CCR2 sulfopeptide (4:1 ratio), revealing the movement of hBD6 resonances toward their positions in the CCR2-bound state (red to orange peak color). The titration of FX in the resulting mixture results in a linear movement of peaks in the $^1$H,$^{15}$N HSQC spectra of hBD6 toward their positions in the FX-bound state (blue to cyan peak color). The titration of FX into hBD6-CCR2 results in a gradual shift of the hBD6 peaks from their positions in the CCR2-bound state to positions in the FX-bound state, demonstrating that heparin competes effectively with CCR2 for hBD6 binding sites. To identify the hBD6 residues most sensitive to CCR2 displacement, we calculated the amide $^1$H,$^{15}$N CSP between hBD6 bound to CCR2 and hBD6 bound to FX (Fig. 8C). Ten hBD6 residues (Phe-1, Phe-2, Asp-3, Glu-4, Cys-6, Cys-27, Gln-28, Lys-29, Ser-30, and Leu-31) displayed CSP differences that are involved in CCR2/FX recognition.

Our results suggest that some of the surfaces occupied by CCR2 sulfo tyrosines may also be utilized for the recognition of the sulfate moieties decorating GAG molecules. A similar competition was also observed to occur between the N-terminal peptide of CXCR4 and heparin (36). The biological relevance of CCR2/FX competition needs further exploration.

**CONCLUSIONS**

In this study, we made a significant contribution to the understanding of how hBD6 could regulates leukocyte migration through its interactions with GAGs by revealing the structural details of how hBD6 binds to GAG, by showing that the binding site of hBD6 displays conformational dynamics on the microsecond-millisecond time scale that may facilitate the selection of the molecular configuration optimal for binding, and by proposing a model for the FX-induced dimerization of hBD6.

Our NMR-derived structural model of the hBD6-FX ternary complex suggests that FX drives dimer formation by interacting parallel to the hBD6-mapped interface, therefore enabling the binding of another hBD6 molecule by a sandwich-like dimerization mechanism. We also demonstrated that high-pressure NMR studies are an additional tool to study the mechanisms of defensin-GAG complex formation. The ability of pressure to dissociate protein assembly has been well documented by various spectroscopies besides NMR (47). Using this approach, we identified, for the first time, a stabilized intermediate stage that could represent a snapshot of the first stage of GAG recognition by hBD6 (Fig. 9). In addition, it is possible that the CCR2 receptor could compete with GAG for hBD6 because these two ligands share the same binding site on the hBD6.

There are only two studies of GAG recognition by $\beta$-defensins: hBD6, in this work, and hBD2 (22). There is a limited sequence homology among the human $\beta$-defensins. As expected, the most conserved residues in the $\beta$-defensin family are the cysteines, glycines, glutamic acid, and basic residues. The identity between hBD2 and hBD6 was observed to be only
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Sandwich-like dimerization intermediate stage

FIGURE 9. Chart representation of sandwich-like dimerization of the hBD6-FX complex. A, binding of FX induces dimerization of hBD6. B, model of hBD6-FX ternary complex dissociation upon application of high-pressure NMR. An intermediate stage of hBD6-FX recognition is proposed.

17%. This result reinforces how difficult it is to identify common regions within the defensins that are responsible for the dimerization process and GAG interaction. Interestingly, the NMR-mapped region for hBD2-heparin interaction encompasses a patch of basic residues (Arg-22 to Gln-26), whereas, in hBD3 to hBD6, this same region contains a conserved acidic residue. This analysis suggests that different residues form the GAG binding site in these defensins, which explains the differential interaction between hBD2 and hBD6 with FX.

The characterization of GAG binding sites remains a complex issue, even for the well-characterized chemokine system. All models of complexes obtained with or without experimental restraints showed that the GAG binding site resides on the surface of the protein. The differences in complex formation may indicate different biological functions between chemokines and defensins. In conclusion, this study shows that hBD6 and GAG are conformationally dynamic, existing as a dynamic ensemble, and that defensin structural plasticity confers an additional layer of mechanistic regulation in the recognition of GAGs.

Understanding the mechanism by which HS interacts with proteins has important implications, particularly for the design of specific HS-derived therapeutic compounds. NMR spectroscopy is a valuable technique for extracting data on protein-HS complexes.

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