Investigation of lysine side chain interactions of interleukin-8 with heparin and other glycosaminoglycans studied by a methylation-NMR approach

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Although the interaction between interleukin-8 (IL-8) and glycosaminoglycans (GAGs) is crucial for the mediation of inflammatory effects, little is known about the site specificity of this interaction. Therefore, we studied complexes of IL-8 and heparin (HEP) as well as other GAGs in a multi-disciplinary approach, involving site-directed mutagenesis, mass spectrometry, fluorescence and solution NMR spectroscopy as well as computer modeling. The interaction between GAG and IL-8 is largely driven by the amine groups of the lysine and the guanidinium groups of arginine side chains. However, due to fast exchange with the solvent, it is typically not possible to detect NMR signals of those groups. Here, we applied reductive 13C-methylation of the lysine side chains providing sensitive NMR probes for monitoring directly the sites of GAG interaction in $^1$H-13C correlation experiments. We focused on the lysine side chains K25, K28, K59, K69 and K72 of IL-8 (1–77), which were reported to be involved in the binding to GAGs. The NMR signals of these residues were assigned in $^1$H-13C HSQC spectra through the help of site-directed mutagenesis, NMR and fluorescence titration experiments in combination with molecular docking and molecular dynamics simulations applied to investigate the involvement of each lysine in the binding with HEP and various GAG hexasaccharides. We identified K25, K69 and K72 to be the most relevant binding anchors of IL-8(1–77) for the analyzed GAGs.

Keywords: chemokine / electrostatics / NMR / protein / glycosaminoglycan interaction / reductive methylation

Introduction

Glycosaminoglycans (GAGs) are complex anionic polysaccharides that occur in various tissues as essential components of the extracellular matrix or on the cellular surface (Gandhi and Mancera 2008; Schiller and Huster 2012). In the past few years, GAGs have also received remarkable attention in tissue engineering and regenerative medicine because of their crucial role in a variety of physiological processes (Prestwich 2011; Senni et al. 2011; Salbach et al. 2012). GAG chains consist of linearly organized repeating disaccharide units of uronic acid and an amino sugar that feature high degrees of heterogeneity concerning their sulfation patterns and glycosidic linkages (Gandhi and Mancera 2008). Generally, it can be distinguished between non-sulfated GAGs such as hyaluronan (HA) and sulfated GAGs including chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), dermatansulfate (DS), keratan sulfate, heparin (HEP) and heparan sulfate (HS). In the latter class, CS and DS contain one sulfate group per disaccharide unit, whereas HEP and HS are higher sulfated with two or three negatively charged groups per subunit (Gandhi and Mancera 2008).

It is known that GAGs interact with a wide range of regulatory proteins like growth factors, adhesion proteins, chemokines and cytokines that are important for several cellular functions such as the organization of the extracellular matrix, the mediation of cell adhesion and migration or the regulation of proliferation and differentiation (Jackson et al. 1991; Krieger et al. 2004; Imberty et al. 2007). Thus, they are also used to functionalize three-dimensional scaffolds for tissue engineering providing an optimal development of the respective tissue (Gandhi and Mancera 2008; Schiller and Huster 2012). As adhesion molecules for pro-inflammatory proteins, GAGs are also involved in immune reactions (Ley et al. 2007). It is reported that due to binding of chemokines to HS, the chemokine gradient along the endothelial surface is stabilized supporting the recruiting of leukocytes. Additionally, binding to HS protects chemokines from proteolytic degradation and enforces their oligomerization, which in turn ensures a high local chemokine concentration close to their receptors increasing the effect on the target cells, the leukocytes (Hoogewerf et al. 1997; Lortat-Jacob et al. 2002). In spite of the high relevance of GAG/protein interactions, relatively few crystal or NMR structures are available so far (Imberty et al. 2007).
the few examples known, HEP and HS have been studied, but very little work has been done with other GAGs.

In the current study, we focus on the interaction of the pro-inflammatory CXC chemokine interleukin-8 (IL-8) with several GAGs including HA, C4S, C6S, DS and HEP. IL-8 plays an important role as a mediator of inflammation by recruiting and activating neutrophils (Baggiolini et al. 1994). In previous work, a common GAG-binding region within the C-terminal α-helix and the proximal N-terminal loop of IL-8 has been identified. In particular, the basic residues H23, K25, K28, K59, R65, K69, K72 and R73 of IL-8(1–77) have been found as binding anchors for the anionic GAGs (Kuschert et al. 1998; Bitomsky and Wade 1999; Krieger et al. 2004; Pichert et al. 2012; Schlorke et al. 2012). But also negatively charged residues such as E75 have been found to respond to GAG binding (Nordsieck et al. 2012). Overall, many structural details of these interactions are largely unknown.

In NMR interaction studies, 1H,15N HSQC titration experiments with GAGs are advantageous as they provide a fingerprint of the entire protein and allow identifying the residues interacting with a specific GAG (Fielding 2007). In such studies, also uncharged or even negatively charged residues have been identified to interact with GAGs as the experiment reports on the impact of the GAG binding on the protein backbone (Fielding 2007). However, the interaction between GAG and protein is to a large degree electrostatics-driven and, therefore, the most interesting groups to look at are the amine groups of the lysine residues and the guanidinium groups of the arginine side chains. However, due to fast exchange with the solvent, it is typically not possible to observe the NMR signals of those groups. A possible solution to this problem is the reductive 13C-methylation of lysine side chains (Means and Feeney 1968; Larda et al. 2012), which generates sensitive [13C]methyl groups at the amino groups and retains the positive charge of the side chain for interaction studies with GAGs.

Here, we have used this approach to study the interaction of the lysine side chains of IL-8(1–77) with various GAG hexasaccharides. Using site-directed mutagenesis, we have assigned those lysine side chains that interact with the GAGs in the 1H,13C HSQC spectra. Fluorescence experiments and molecular docking in combination with molecular dynamics (MD) simulations were applied to provide a more detailed molecular picture of the interaction of IL-8 with various GAGs and to gain further information about the role of each lysine residue for binding GAGs.

Results

In this study, we investigated the interaction of the lysine residues of IL-8(1–77) with GAGs by reductive methylation of the lysine side chains followed by NMR analysis (Larda et al. 2012). In order to assign the 1H,13C HSQC spectra of methylated IL-8(1–77), a series of single mutations was prepared, in which each lysine residue that was previously reported to interact with GAGs were recombinantly expressed as fusion proteins in Escherichia coli. To introduce all point mutations at the respective position, site-directed mutagenesis was used. After expression and purification of the fusion proteins by affinity chromatography on chitin beads, the target proteins were cleaved from the beads by dithiothreitol (DTT) to obtain protein thioesters that are not stable to hydrolysis and were easily converted into the respective native proteins. The entire expression, purification, and cleavage procedure was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) and reversed phase-high performance liquid chromatography (RP-HPLC). To refold the denatured proteins, stepwise dialysis was applied (Nordsieck et al. 2012) and all proteins were finally purified by preparative RP-HPLC. Correct identity of the proteins were confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), revealing a single charged peak at the expected average m/z of 8919.8 (theoretical mass: 8918.4 Da) and a purity of >95% for all variants was detected by RP-HPLC (Supplementary data, Table S1). As an example, the mass spectrum and the chromatogram of refolded and purified K72Q(IL-8(1–77)) are shown in Figure 1A.

Receptor activity studies of the KxQ-IL-8(1–77) variants

The ability of the KxQ-IL-8(1–77) variants to activate the CXC receptor 1 was tested by intracellular inositol phosphate (IP) accumulation assay (Figure 1B). To this end, cell line derived from monkey kidney tissue (COS-7) cells were transiently co-transfected with the CXC receptor 1 and a chimeric G protein (GαQ(Gsa4myr) that switches the Gt to the Gq signaling pathway and thereby allowing a very sensitive detection of the IP levels by a radioactive technique. After stimulation and anion-exchange chromatography, intracellular IP levels were determined by scintillation measurements. Notably, all KxQ-IL-8(1–77) variants showed similar efficacy to the wild-type (WT) IL-8(1–77) revealing that all variants activate the receptor in the same manner. Moreover, for most KxQ-IL-8(1–77) variants, comparable potencies with that of the WT (9.3 nM) were determined (Table 1). Only K25Q-IL-8(1–77) showed a slightly increased potency of 2.5-fold compared with WT. This demonstrates that most recombinantly synthesized variants are capable to activate the CXCR1 equivalent to WT IL-8(1–77), and thus, the biological activity of the variants is not affected by the exchange of the respective amino acid.

After methylation of IL-8(1–77), the protein can still activate the CXC receptor; however, the EC50 value increases ~9-fold (82.5 vs. 9.3 nM) compared with the WT. This can be related to the altered structure of the amine group at the lysine residues, which may slightly modify the receptor access and binding pose of IL-8(1–77). Nevertheless, methylated IL-8(1–77) activates the CXCR1 receptor and shows an efficacy of >80% of the WT IL-8(1–77).

[13C]methyl labeling of IL-8(1–77) and assignment of the 1H,13C HSQC NMR spectra

To observe the lysine side chains of IL-8(1–77) in interaction studies with GAGs in the NMR spectra, we selectively labeled them with [13C]methyl groups by reductive methylation (Means and Feeney 1968; Larda et al. 2012). The incorporation of [13C]methyl groups into IL-8(1–77) or its variants was
confirmed by MALDI-TOF MS and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) after tryptic digestion. A mass shift between unmethylated IL-8(1–77) (M_{av,exp} = 8919.8 ± 2.7 Da, M_{av,theo} = 8918.4 Da) and the methylated IL-8(1–77) (M_{av,exp} = 9201.3 ± 3.6 Da, M_{av,theo} = 9199.0 Da) of 282 ± 6 amu could be determined, which corresponds to the addition of 20.2 ± 0.4 methyl groups indicating that in total all nine lysines as well as the free N-terminus are dimethylated (Supplementary data, Figure S1). For further site-specific analysis, the methylated protein was digested with trypsin and the peptide mix subjected to a nano-HPLC/nano-ESI MS/MS analysis. In this analysis, a complete sequence coverage could be obtained and the dimethylation of all nine internal and the N-terminal positions was positively confirmed (Supplementary data, Table S2).

Table I. EC50 values of the CXC receptor 1 activation by WT and methylated IL-8(1–77) as well as the KxQ-IL-8(1–77) variants

<table>
<thead>
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<th>IL-8(1–77) variant</th>
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<th>pEC50 ± SEM</th>
</tr>
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<tbody>
<tr>
<td>IL-8(1–77)</td>
<td>9.5</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>K25Q-IL-8(1–77)</td>
<td>26.2</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>K28Q-IL-8(1–77)</td>
<td>13.4</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>K59Q-IL-8(1–77)</td>
<td>9.6</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>K69Q-IL-8(1–77)</td>
<td>15.5</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>K72Q-IL-8(1–77)</td>
<td>10.5</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>methyl-IL-8(1–77)</td>
<td>82.5</td>
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EC50 values were determined from 2 to 10 independent experiments, each performed in triplicate.

Next, we acquired NMR spectra of the methylated IL-8. A 1H-{\textsuperscript{13}C} HSQC spectrum of [\textsuperscript{13}C]methyl-IL-8(1–77) containing nine [\textsuperscript{13}C]DMLs and the [\textsuperscript{13}C]dimethyl-N-terminus. The lysines K25, K28, K59, K69 K72 and the N-terminus are assigned to the corresponding peaks. Four lysine residues remain unassigned.

Fig. 2. 1H-{\textsuperscript{13}C} HSQC NMR spectrum with the assignment of several [\textsuperscript{13}C]dimethylamine peaks of [\textsuperscript{13}C]methyl-IL-8(1–77) containing nine [\textsuperscript{13}C]DMLs and the [\textsuperscript{13}C]dimethyl-N-terminus. The lysines K25, K28, K59, K69 K72 and the N-terminus are assigned to the corresponding peaks. Four lysine residues remain unassigned.

Fig. 1. (A) Exemplary characterization of the K72Q-IL-8 variant by MALDI-TOF mass spectrometry and RP-HPLC. In the mass spectrum, the average mass was detected to be 8920 Da, which agrees well with the theoretical average mass of 8919 Da. The peak at m/z 4460 corresponds to the double charged compound (z = 2). In the RP-HPLC chromatogram, the variant was monitored using a gradient of 20–70% acetonitrile (0.08% trifluoro acetic acid (TFA)) in water (0.1% TFA) in 40 min. (B) Characterization of the biological activity of the KxQ-IL-8 variants by an IP accumulation assay in cell line derived from monkey kidney tissue cells. EC50 values are given in Table I.

Table I. EC50 values of the CXC receptor 1 activation by WT and methylated IL-8(1–77) as well as the KxQ-IL-8(1–77) variants

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<td>K59Q-IL-8(1–77)</td>
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<td>methyl-IL-8(1–77)</td>
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EC50 values were determined from 2 to 10 independent experiments, each performed in triplicate.
Residues K25 and K28 were straightforward to assign as these peaks clearly disappeared in the spectra of the variants (Supplementary data, Figure S2A and B). Residues K69 and K72 belong to a cluster of overlapping peaks attributed to solvent-exposed, highly mobile lysines. Thus, they could not be assigned separately (Supplementary data, Figure S2D and E). By increasing the pH value up to 10.5 in the protein solution, the dispersion of the $[^{13}\text{C}]$dimethyllysine (DML) was improved and we could observe more than four peaks belonging to the cluster of overlapping peaks (Supplementary data, Figure S3). Despite the improved peak dispersion, the subsequent interaction studies of IL-8(1–77) and GAGs had to be carried out at a physiological pH of 7 to provide correct protein folding and intermolecular interactions. Therefore, the dispersion of the signals from K69 and K72 could not further be improved. Interestingly, two peaks, which appear reflection symmetrically at the same $^1\text{H}$ frequency, could be assigned to K59 indicating a restriction in movement of this side chain. Nevertheless, both peaks are well separated and, therefore, straightforward to assign.

Additionally, the $[^{13}\text{C}]$dimethyl-N-terminus could also be assigned on the basis of thrombin cleavage. In the NMR spectrum of the cleavage product, the NMR signal of the methylated N-terminus was significantly attenuated (Supplementary data, Figure S4B). This is the result of incomplete thrombin cleavage as revealed by MALDI-TOF MS (Supplementary data, Figure S4A).

$^1\text{H}–^{13}\text{C}$ HSQC NMR titration studies

To investigate the impact of different GAG hexasaccharides on the lysine side chains of IL-8(1–77), NMR titration experiments were carried out. A representative titration with HEP is shown in Figure 3A illustrating the superposition of two $^1\text{H}–^{13}\text{C}$ HSQC spectra representing $[^{13}\text{C}]$methyl-IL-8(1–77) in the absence (black) and in the presence of 0.33 mM HEP (red). Clearly, the NMR signals of the side chains of K25, K59, K69 and K72 show pronounced chemical shift changes indicative for an interaction with HEP. The $^1\text{H}–^{13}\text{C}$ HSQC NMR spectra of IL-8 in the absence and in the presence of various GAGs are shown in Supplementary data, Figure S5. In order to map out the lysine side chains that show strongest interaction with the respective GAG, weighted chemical shift perturbation charts were plotted (Figure 3B). The experimental error in the determination of these chemical shift changes is on the order of 0.01 ppm, also a threshold of significance of 0.01 ppm was applied. Clearly, not all GAGs investigated in this study show an interaction with lysine side chains of IL-8(1–77). There is no chemical shift change of the lysine side chains in $[^{13}\text{C}]$methyl-IL-8(1–77) upon titration with the non-sulfated HA. Although it is reported that HA interacts with IL-8(1–77) (David et al. 2008), the interaction proceeds apparently without larger structural alterations concerning lysine side chains, which is in agreement with the observation that also the IL-8(1–77) backbone does not show much perturbation when HA is added (Pichert et al. 2012).

Stronger effects were observed when IL-8(1–77) was titrated with C4S, C6S, DS and HEP illustrating the electrostatic interaction of the negatively charged sulfate group of the GAG with the positively charged lysine side chains of IL-8(1–77). The perturbation charts point out that the most highly sulfated HEP has the most extensive effect on the lysine side chains, followed by the GAGs with a single sulfate group. Strongest chemical shift changes are observed for K69 and K72, whereas K25 showed the largest $^{13}\text{C}$ chemical shift change upon titration with HEP. Also, the split signals of K59 reflect the binding of HEP and C6S. Overall, chemical shifts obtained from these NMR titration experiments are moderate, but demonstrate well a crucial role for the side chains of K25, K59, K69 and K72 during GAG binding, which is in agreement with NMR data on the response of the IL-8(1–77) backbone to GAG binding (Kuschert et al. 1998; Pichert et al. 2012).

Fluorescence titration studies

The perturbations of the chemical shifts in the NMR results indicated that K25, K69 and K72 are the most crucial side chains for GAG interaction. With the set of single residue mutations, produced for this study, we investigated if abolishing these positive charges would result in a weaker GAG/IL-8(1–77) interaction. To this end, tryptophan fluorescence titration studies of IL-8(1–77) and the respective variants with HEP hexasaccharides were carried out and $K_D$ values for the interaction determined.
ate 2-fold increase in these positions con-"return"
cerning the chemical shift changes observed for the methylated lysines in variants K25Q, K69Q and K72Q in agreement with the large GAG binding. The K59Q-IL-8(1–77) is the particular, we determined the binding free energies of HEP to simulations of IL-8(1–77) variant showed a moderate contribution to the interaction energy, while the other lysines do not seem to contribute much to GAG binding. The impact of individual lysine residues and of methylation on GAGs binding in terms of the total binding energy varies for different GAGs (Supplementary data, Table S3).

Discussion
NMR spectroscopy is a very powerful method to study intermolecular interactions and in particular protein/ligand equilibriums (Fielding 2007). One standard approach involves \(^{15}\)N labeling of the protein and subsequent detection of \(^{1}H-{^{15}}N\) HSQC spectra with varying ligand concentrations (Fielding 2007). Although this method allows to study the influence of a ligand on all protein backbone amides (except proline), the fast solvent exchange of the amine and guanidinium side chains typically prevents the observation of these groups. However, for the interaction of proteins with negatively charged GAGs, these side chains play an important role as the interaction is largely of electrostatic origin (Imberty et al. 2007). One possibility to study the lysine side chains is reductive methylation (Means and Feeney 1968). Under these conditions, \(^{13}\)C-methyl groups are covalently bound to the -NH\(_3\) of lysine side chains and the α-NH\(_2\) of the N terminus. Thus, sensitive probes for the investigation of the side chain–GAG interaction are generated, which can conveniently be observed in simple \(^{1}H-{^{13}}C\) HSQC NMR experiments. Although the methylation represents an alteration of the protein structure, the electrostatic nature of the amine is retained and the interaction of these groups with the sulfated GAG chains can be observed. Nevertheless, it should be considered that methylation may interfere with the hydrogen bond pattern and methylated lysine groups may be involved in cation–π interactions (Daze and Hof 2013). Under these conditions, it is possible to investigate small structural changes of the side chains possibly induced by electrostatics or hydrogen bonding (Bokoch et al. 2010; Künze et al. 2012). Thus, the method can provide relevant information about the involvement of the lysine side chains of IL-8(1–77) in the interaction with GAGs.

The interactions between IL-8(1–77) and various GAGs have been in the focus of several previous studies. In particular basic residues within the C-terminal α-helix and the proximal N-terminal loop of IL-8(1–77) were determined as binding sites for the sulfate groups of anionic GAGs (Kuschert et al. 1998; Bitomsky and Wade 1999; Lortat-Jacob et al. 2002; Krieger et al. 2004; Pichert et al. 2012). As there are no co-crystals available for the IL-8/GAG complex, NMR remains the only experimental method that provides atomistic resolution. The lysine side chains K25, K28, K59, K69 and K72 of IL-8(1–77) could be identified as interaction partners of various GAGs by analyzing protein backbone chemical shift alterations (Kuschert et al. 1998; Pichert et al. 2012). Here, we focus directly on the interaction of these side chains with various GAGs using the lysine methylation approach. As the \(^{13}\)C-methyl groups of the lysine side chains

### Table II. Dissociation constants (\(K_D\)) of WT and methylated IL-8(1–77) and its variants during binding HEP hexasaccharides obtained from Trp fluorescence titration studies at a protein concentration of 1 µM at 25°C

<table>
<thead>
<tr>
<th>IL-8(1–77) variant</th>
<th>(K_D \pm SD/\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8(1–77)</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>K25Q-IL-8(1–77)</td>
<td>10.9 ± 3.0</td>
</tr>
<tr>
<td>K28Q-IL-8(1–77)</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>K59Q-IL-8(1–77)</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>K69Q-IL-8(1–77)</td>
<td>10.9 ± 5.0</td>
</tr>
<tr>
<td>K72Q-IL-8(1–77)</td>
<td>11.5 ± 2.6</td>
</tr>
<tr>
<td>Methyl-IL-8(1–77)</td>
<td>2.2 ± 0.4</td>
</tr>
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</table>

\(K_D\) values were determined from 3 to 5 independent experiments.

![Graph](attachment:image)

**Fig. 4.** Contribution of each methylated lysine residue to the binding-free energies (\(ΔG\)) of IL-8(1–77) during binding of HEP hexasaccharides as determined from MM-GBSA calculations.

(Table II). The first interesting result is that native and methylated IL-8(1–77) feature the same \(K_D\) for HEP binding within experimental error. This indicates that the methylation of the lysine residues does not significantly alter the interaction with various GAGs confirming the validity of the NMR approach used here. Further, residue K28 does not seem to be crucial for the interaction of IL-8(1–77) with GAGs as the WT and the mutated K28Q-IL-8(1–77) variant showed the same \(K_D\) value. In contrast, the \(K_D\) for GAG binding increased about 5-fold for the IL-8(1–77) variants K25Q, K69Q and K72Q in agreement with the large chemical shift changes observed for the methylated lysines in these positions confirming the central role of these residues for GAG binding. The K59Q-IL-8(1–77) variant showed a moderate 2-fold increase in \(K_D\) upon HEP titration, which agrees with the moderate chemical shift changes observed for this residue upon HEP binding.

**MD simulations**
To complement our experimental studies, we carried out MD simulations of IL-8(1–77)/HEP hexasaccharide complexes. In particular, we determined the binding free energies of HEP to methyl-IL-8(1–77) and IL-8(1–77). According to our simulations, the binding free energy of HEP hexasaccharide to IL-8(1–77) is −45.1 kcal/mol, while complete lysine methylation of IL-8 (1–77) reduces this value to −36.6 kcal/mol. Further, in the simulations, the relative contribution of each lysine residue to HEP binding was calculated (Figure 4). These results are in agreement with the chemical shift changes and the \(K_D\) values of the interaction of the IL-8(1–77) variants with HEP: K25, K69 and K72 show the strongest contribution to the interaction energy, while the other lysines do not seem to contribute much to GAG binding. The impact of individual lysine residues and of methylation on GAGs binding in terms of the total binding energy varies for different GAGs (Supplementary data, Table S3).
represent isolated spin systems, a sequential assignment of these groups is not straightforward. Therefore, the assignment of the most relevant lysine residues was accomplished by site-directed mutagenesis. Due to the lysine-to-glutamine exchange, the respective \(^1\text{H}\)\(^{13}\text{C}\)DML peak disappears in the \(^1\text{H}\)^{13}\text{C}\)HSQC NMR spectrum and can thus be assigned.

The titrations with various GAGs confirmed the importance of these lysine side chains for the interaction with the sulfated sugars. In particular, K69 and K72 were identified as the lysine side chains with the most important contribution to GAG binding. In addition, K25 shows a very strong effect when binding HEP, which appears to be related to the unique -NHSO\(_3\) group of the amino sugar of HEP, which no other GAG used in this work showed. K25 also seems to be the most crucial lysine residue for the activation of the CXC receptor 1 by IL-8(1–77) (Table I). This can be concluded from the observation that the mutation of K25 to glutamine also resulted in the strongest increase in the \(\text{EC}_{50}\) value for receptor activation. In addition to the largest chemical shift changes observed for the methylated version of these lysine side chain, single K25Q variants of IL-8 (1–77) showed a 6-fold higher \(K_D\) value for HEP binding (Table II). Large chemical shift alterations (Figure 3) and much increased \(K_D\) values for HEP binding (Table II) were also observed for K69 and K72 and the mutants, respectively, confirming the important role of these side chains in the interaction with GAGs. This could further be confirmed by MD simulations that allowed calculating the contribution of each of those residues to HEP binding (Figure 4) and other GAGs (Supplementary data, Table S3). Clearly, these residues provided the largest contribution to the binding-free energy of HEP.

A more moderate effect of GAG binding on K59 was indicated by weak chemical shift alteration of this residue. In agreement with the NMR data, the \(K_D\) of the K59Q variant of IL-8 (1–77) only showed a 2-fold increase for HEP binding compared with the WT and the contribution of this residue to the binding-free energy of HEP was less than \(-1\) kcal/mol. This suggests that K59 is less strongly involved in GAG binding most likely due to long-range electrostatics. More specifically, only the GAGs that feature a C6-sulfation on the amino sugar (C6S and HEP) induced a small chemical shift change. This difference can be illustrated for the different interaction patterns of C4S and C6S: C4S induces chemical shift changes of K69 and K72, but no chemical shift change of K59; in contrast, C6S induced chemical shift changes of K59 but a smaller effect on K69 and K72. This agrees with previous investigations of the IL-8/GAG interaction (Nordsieck et al. 2012; Pichert et al. 2012). Nevertheless, these differences in the chemical shift changes of C6S for K59 and K69/K72 are within experimental error and are also not exactly reproduced in the computer simulation (Supplementary data, Table S3).

An interesting feature of residue K59 is that two signals with identical \(^1\text{H}\) but different \(^{13}\text{C}\) chemical shifts have been detected. Such a phenomenon has been known from literature (Bokoch et al. 2010). One could hypothesize that an electrostatic interaction with a proximal negatively charged residue is the reason for the splitting of the K59 signal into two peaks. This assumption could be confirmed by computer simulation. Supplementary data, Figure S7 shows the structure adopted by the methylated K59 residue during the 10 ns MD simulation, in which the different conformations of the dimethylated side chain can be observed distributed in two clusters. The MD simulations show that the K59 side chain moves sometimes randomly and totally unrestricted and sometimes directed toward the proximate negatively charged aspartic acid D57 forming a temporary salt bridge. Due to the salt bridge, the side chain is temporally restricted in its movement, explaining why the influence of the chemical environment is not averaged leading to the occurrence of two peaks in the \(^1\text{H}\)^{13}\text{C}\)HSQC NMR spectrum for this residue. Additionally, it could be shown that the two peaks merge under conditions of increased pH, presumably as a result of weakening of the temporary salt bridge (Supplementary data, Figure S3A). Although the two signals likely reflect two different conformations of K59, rather similar influences on the chemical shift of both peaks occur when binding C6S or DS. Although ligands typically have a stronger affinity for one of the conformers, the undirected electrostatic nature of the interaction appears to average out any possible difference in this situation.

The last Lys residue that was investigated here is K28. This side chain did not show any chemical shift change upon titration with GAGs (Figure 3). Furthermore, the mutation K28Q resulted in the same \(K_D\) for HEP binding of IL-8(1–77) (Table II), and the energetic contribution of this residue to HEP binding was marginal as revealed by MD computer simulation (Figure 4). MD simulation could further show that K28 interacts with the negatively charged residues E29 and D50 in close proximity, which also influences the dynamic behavior of K47. Therefore, K28 plays a role in the establishment of an electrostatic environment in this region and also possibly in the orientation of K25, which according to our data is one of the anchoring residues for GAG binding.

Finally, the other Lys residues of IL-8 (i.e. K8, K16, K20, K47), which were not investigated directly, did not show any chemical shift alterations in the HSQC spectra and the computer experiments also did not reveal any significant contribution of these residues to GAG binding. Further, in agreement with the modeling results, an involvement of the N terminus in GAG binding is unlikely as also discussed for other chemokines (Ziarek et al. 2013).

A last question concerns the impact of the methylation on protein structure. Several x-ray crystallography and NMR investigations confirmed that reductive methylation does not significantly alter protein structure (Rayment 1997; Walter et al. 2006; Bokoch et al. 2010) and does not cause unwanted protein–protein interactions (Rypniewski et al. 1993). In addition, we carried out several assays to confirm that methylated IL-8(1–77) shows very close resemblance to the WT protein. We also investigated the biological activity of the methylated IL-8(1–77). The \(\text{EC}_{50}\) value of methylated IL-8(1–77) determined by an IP accumulation assay was clearly increased by a factor of 9 compared with the WT. Although the CXC receptor 1 was still activated by methylated IL-8(1–77), this result indicates that the methylation interferes with optimal receptor activation. Nevertheless, this small protein modification did not interfere with the capability of methylated IL-8(1–77) to interact with GAGs. Further, \(^1\text{H}\) NMR spectra of the methylated and native IL-8(1–77) were comparable, indicating that the general tertiary structure of the molecule was not compromised by the methylation (Supplementary data, Figure S6).
Conclusions

We have investigated in detail the interaction of the lysine side chains of IL-8(1–77) with HEP and other GAG hexasaccharides using reductive $^{13}$C-methylation in combination with $^{3}$H-$^{13}$C HSQC NMR spectroscopy. The incorporated $[^{13}C]$methyl groups represent sensitive NMR probes for monitoring directly the sites of GAG interaction without any appreciable influence on the binding strength. Furthermore, we could demonstrate that the combination of NMR and fluorescence spectroscopy with MD simulation is very effective for the investigation of complex systems such as GAG/protein complexes. These complementary approaches enable the identification of lysine residues as binding anchors of IL-8(1–77) for the analyzed GAGs. In particular, the side chains of K25, K69 and K72 are involved in GAG binding. K59 only shows a small response in the interaction of IL-8(1–77) with GAGs that feature a C6 sulfation. A structural model displaying the interaction of the Lys side chains of the IL-8 dimer with HEP is shown in Figure 5. Our results might be important for both the understanding of fundamental biological processes and the design of biocompatible scaffold materials in the field of tissue engineering and regenerative medicine that feature GAG derivatives as binding sites for regulatory proteins and mediators.

Materials and methods

DNA constructs

DNA polymerases, restriction enzymes, nucleoside triphosphates and primers for cloning were purchased from Thermo Scientific (St Leon-Roth, Germany). The cDNA encoding IL-8 (1–77) was amplified by polymerase chain reaction (PCR) from a vector containing the IL-8 cDNA using the IL-8 forward primer 5'-GGTGGTGATATGGCAGTTTTGCCAAGGAGTG C-3' including an NdeI-restriction site and the IL-8(1-77) reverse primer 5'-GAGCAATTCTAGGCTTCCGATGAATTCTCAGCCCTCTTCAA-3'.

Protein preparation

All KxQ-IL-8(1–77) variants (x represents the mutated amino acid position in the protein sequence: 25, 28, 59, 69, 72) were expressed as fusion proteins in the pTXB1 vector, C-terminally linked to the mycobacterium xenopi (Mxe) intein and a chitin-binding domain, in order to use the IMPACT™-system from New England Biolabs (Chong et al. 1997). The fusion proteins were expressed in transformed E. coli ER2566 bacteria cells at 37°C, and the expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside. After 6 h, the cells were harvested by centrifugation, re-suspended in column buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (pH 8.0) and 37°C, and the expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside. After 6 h, the cells were harvested by centrifugation, re-suspended in column buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (pH 8.0) and cell lysis was performed by French® Press and ultrasonic pulses. All buffer ingredients were purchased from Sigma-Aldrich (Steinheim, Germany). The released DNA was degraded by DNase I (AppliChem, Darmstadt, Germany) in the presence of 4 mM MgCl2. After centrifugation, the lysates were separated and the inclusion bodies were washed with column buffer containing 2 M urea and subsequently with column buffer. They were solubilized twice with column buffer containing 8 M urea. The preparations of the thioesters were performed as described previously (Nordsieck et al. 2012). For equilibration of the chitin beads (New England Biolabs), the beads were treated differently. Depending on the loaded solution (lysate or solubilise), column buffer or column buffer containing 2 M urea was used. Afterwards, lysate or solubilise were loaded onto the respective column. All columns were washed and the cleavage was induced by the addition of cleavage buffer (column buffer or 2 M urea column buffer, both containing 0.1 M DTT, Thermo Scientific). Expression and purification were monitored by SDS–PAGE and RP-HPLC.

Hydrolisis of the KxQ-IL-8(1–77) thioesters was performed by adding NaOH to obtain a pH of 9.5 and the solutions incubated overnight at 4°C. Afterwards, the KxQ-IL-8(1–77) variants were refolded by stepwise dialysis against three refolding buffers (Nordsieck et al. 2012). Purification of the refolded KxQ-IL-8(1–77) variants was carried out by preparative RP-HPLC using a gradient of 10–50% acetonitrile (ACN) (0.08% trifluoro acetic acid (TFA)) in water (0.1% TFA) over 40 min. The identities of the variants were confirmed by MALDI-TOF MS and the purities were determined by RP-HPLC. WT IL-8(1-77) was recombinantly expressed, purified and refolded as described previously (Pichert et al. 2012).
Glycosaminoglycan-Lys side chain interactions of IL-8

Cell culture and IP accumulation assay
For the IP accumulation assays, COS-7 cells were maintained and treated as described previously (Nordsieck et al. 2012). In brief, the cells were seeded into 48-well plates (45,000 cells/well) and transiently cotransfected with 0.32 µg plasmid DNA encoding the chemokine receptor CXCR1 and the enhanced green fluorescent protein and 0.08 µg plasmid DNA coding for the chimera G protein Gzα6q4i4myr using METAFECTENE.eGFP served as a transfection control (David et al. 2008). After stimulation, the assay was performed as described by Nordsieck et al. (2012).

[^13]C methyl labeling of IL-8(1–77) and its variants
Reactive[^13]C methylation was performed as described in literature (Means and Feeney 1968; Künze et al. 2012). Borane–ammonia complex (NH3 × BH3; Sigma-Aldrich) used as reducing agent and[^13]C formaldehyde (Cambridge Isotope Laboratories, Andover, USA) were added sequentially in excess to the purified protein. The reaction was stopped by adding glycine. Undesired reaction products as well as methylation reagents were removed by extensive dialysis against buffer containing 20 mM phosphate and 50 mM NaCl (pH 7.0). Reductive methylation was monitored by MALDI-TOF MS as well as LC-ESI-MS/MS after tryptic digestion (Supplementary data).

All NMR studies were carried out on Bruker DRX 600 and Avance III 600 NMR spectrometers (Bruker BioSpin, Rheinstetten, Germany) using a 5 mm triple-inversion probe at 30°C. NMR samples contained IL-8(1–77) or its variants in following concentrations:[^13]C methyl-IL-8(1–77) (0.84 mM),[^13]C methyl-K25Q-IL-8(1–77) (0.72 mM),[^13]C methyl-K28Q-IL-8(1–77) (0.62 mM),[^13]C methyl-K59Q-IL-8(1–77) (0.55 mM),[^13]C methyl-K69Q-IL-8(1–77) (1.06 mM),[^13]C methyl-K72Q-IL-8(1–77) (1.02 mM) and[^13]C methyl-IL-8(6–77) (0.50 mM). The protein was dissolved in 0.5 mL 90% H2O/10% D2O containing 20 mM phosphate and 50 mM NaCl (pH 7.0).[^1]H NMR spectra were acquired using a WATERGATE W5 pulse sequence (Liu et al. 1998) for water suppression and a globally optimized alternating phase rectangular pulse for[^13]C decoupling.[^1]H-[^13]C HSQC NMR spectra were recorded using the HSQC pulse sequence of Mori et al. (1995). For each increment, 16 scans were collected with spectral widths of 9.6 kHz for[^1]H and 1.2 kHz for[^13]C as well as 128 data points in the[^13]C dimension. NMR titrations were carried out by successively adding aliquots of a solution with concentrated GAG hexasaccharides to[^13]C methyl-IL-8(1–77) and recording a[^1]H-[^13]C HSQC NMR spectrum. For the titration, equal volumes of GAG solution from the stock solutions (10 mg/mL for HA, 5 mg/mL for the other GAGs) were added to keep the dilution effect approximately constant. Furthermore, varying volumes of protein solution were used, resulting in some differences in the molar concentration of the individual titration steps. Weighted chemical shift changes were calculated according to Cavanagh et al. (1996)

\[
\Delta \delta(^1H, ^{13}C) = \sqrt{(\Delta \delta_1)^2 + \left(\frac{\Delta \delta_2}{4}\right)^2},
\]

where Δδ_H and Δδ_C represent the changes of the chemical shifts in the[^1]H and[^13]C dimension, respectively.

Proteolytic cleavage of[^13]C methyl-IL-8(1–77)
For the cleavage of the N-terminus of[^13]C methyl-IL-8(1–77), the protein was digested as described by Mortier et al. (2008) using the serine protease thrombin (Sigma-Aldrich, St Louis, USA), which was added in two steps to the protein solution. The incubation was carried out at 37°C for 15 h with an enzyme–substrate molar ratio of 1/100 and for additional 9 h with an enzyme–substrate molar ratio of 1/50. The proteolytic cleavage of[^13]C methyl-IL-8(1–77) was terminated by the addition of TFA. The N-terminally truncated protein was dialyzed twice against buffer containing 20 mM phosphate and 50 mM NaCl (pH 7.0). Cleavage of the[^13]C methylated-N-terminus was controlled by MALDI-TOF MS.

Fluorescence titration studies
Tryptophan fluorescence titration experiments were carried out on a Fluoromax 2 spectrometer (JobinYvon, Edison, NJ, USA) using an excitation wavelength of 290 nm. Emission was measured from 300 to 470 nm at 25°C. A solution with HEP hexasaccharides was added successively to 1 µM IL-8(1–77) or its variants presented in buffer containing 20 mM phosphate and 50 mM NaCl (pH 7.0). Fluorescence intensities at the emission maximum were recorded as a function of GAG concentration to analyze the binding affinity. Dissociation constants were determined as described in literature (Ladokhin et al. 2000; Theisgen et al. 2011).

Computer modeling and MD
For modeling, the x-ray structure of monomeric IL-8(1–77) (PDB ID 3IL8, 2.00 Å) was used. The structures of hexameric HA, C4S, C6S, DS and HEP were modeled in MOE (Chemical Computing Group, Inc. 2011). The binding poses of the GAGs on WT IL-8(1–77) were taken from previous docking calculations (Pichert et al. 2012). These complexes were further used for modeling the IL-8(1–77) GAG complexes, in which all lysine residues were dimethylated.

The non-standard amino acid DML was parameterized to be compatible with AMBER 11 (Case et al. 2010) using RESP charges obtained with R.E.D. III program (Duprèade et al. 2010). Sulfate charges for GAG residue libraries were obtained from literature (Huige and Altona 1995). Each complex was solvated in a truncated octahedron TIP3P water periodic box and neutralized by counterions. MD simulations were preceded by two energy minimization steps: 500 cycles of steepest descent and 1000 cycles of conjugate gradient with 10 kcal/(mol Å) harmonic restraints on protein atoms; 3000 cycles of steepest descent and 3000 cycles of conjugate gradient without constraints; 10 ps of heating of the system from 0 to 300 K; 30 ps of MD equilibration at 300 K and 106 Pa in isothermal isobaric ensemble (NPT). 10 ns of productive MD runs were carried out in periodic boundary conditions in the NPT ensemble with Langevin temperature coupling with collision frequency parameter γ = 1 ps⁻¹ and Berendsen pressure coupling with a time constant of 1.0 ps. The SHAKE algorithm, a 2 fs time integration step, an 8 Å cutoff for non-bonded interactions and the Particle Mesh Ewald method were used. MD
trajectories were recorded each 2 ps. The ff99SB force field parameters for the protein and the GLYCAM06 for the GAGs were used. For each complex, one unconstrained MD simulation and one with 10 kcal/(mol Å) harmonic constraints on IL-8 (1–77) backbone atoms were carried out. When unrestrained, IL-8(1–77) fold could experience some distortions, which might be caused by instability of the force field for this small fold as previously discussed in the literature (Kadirkar et al. 2008). All the obtained trajectories, in which the GAGs remained bound in the initial binding mode, were taken for the further analysis, and the results represent the data from the unrestrained and restrained MD simulations.

Free energy calculations
Energetic post-processing of the trajectories and per residue energy decomposition were done in a continuous solvent model using MM-GBSA with igb = 2.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Abbreviations
ACN, acetonitrile; CAS, chondroitin-4-sulfate; C6S, chondroitin-6-sulfate; COS-7, cell line derived from monkey kidney tissue; DML, dimethyllysin; DS, dermatansulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GAG, Glycosaminoglycan; HA, Hyaluronan; HEP, heparin; HS, heparan sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-8, interleukin-8; IP, inositol phosphate; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MD, molecular dynamics; NPT, isothermal isobaric ensemble that maintains constant number of particles N, constant pressure and constant time; PCR, polymerase chain reaction; RP-HPLC, reversed phase-high performance liquid chromatography; SDS-Page, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoro acetic acid; WT, wild-type.

References


