Heparin Oligosaccharides Inhibit Chemokine (CXC Motif) Ligand 12 (CXCL12) Cardioprotection by Binding Orthogonal to the Dimerization Interface, Promoting Oligomerization, and Competing with the Chemokine (CXC Motif) Receptor 4 (CXCR4) N Terminus

Received for publication, June 20, 2012, and in revised form, November 5, 2012. Published, JBC Papers in Press, November 12, 2012, DOI 10.1074/jbc.M112.394064

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Background: GAG/CXCL12 interactions are critical for chemokine function but co-administration may abrogate their individual cardioprotective effects in a clinical setting.

Results: Biophysical studies distinguish CXCL12 residues involved in dimerization from those likely to contact heparin directly.

Conclusion: CXCL12 dimerization is required for high affinity heparin binding and protects N-terminal degradation.

Significance: Knowledge of the GAG-binding site will enable future development of heparin-insensitive CXCL12 therapeutics.

The ability to interact with cell surface glycosaminoglycans (GAGs) is essential to the cell migration properties of chemokines, but association with soluble GAGs induces the oligomerization of most chemokines including CXCL12. Monomeric CXCL12, but not dimeric CXCL12, is cardioprotective in a number of experimental models of cardiac ischemia. We found that co-administration of heparin, a common treatment for myocardial infarction, abrogated the protective effect of CXCL12 in an ex vivo rat heart model for myocardial infarction. The interaction between CXCL12 and heparin oligosaccharides has previously been analyzed through mutagenesis, in vitro binding assays, and molecular modeling. However, complications from heparin-induced CXCL12 oligomerization and studies using very short oligosaccharides have led to inconsistent conclusions as to the residues involved, the orientation of the binding site, and whether it overlaps with the CXCR4 N-terminal site. We used a constitutively dimeric variant to simplify the NMR analysis of CXCL12-binding heparin oligosaccharides of varying length. Biophysical and mutagenic analyses reveal a CXCL12/heparin interaction surface that lies perpendicular to the dimer interface, does not involve the chemokine N terminus, and partially overlaps with the CXCR4-binding site. We further demonstrate that heparin-mediated enzymatic protection results from the promotion of dimerization rather than direct heparin binding to the CXCL12 N terminus. These results clarify the structural basis for GAG recognition by CXCL12 and lend insight into the development of CXCL12-based therapeutics.

Cardiovascular disease is the leading cause of death in the United States, and greater than 50% of these fatalities are asymptomatic (1). The lack of warning for acute cardiovascular injuries, such as myocardial infarctions, underscores the need for highly effective clinical therapeutics. To reduce the possibility of thrombosis at the site of infarction, the American College of Cardiology and the American Heart Association recommend the administration of anticoagulant drugs, such as heparin, during medical treatment for myocardial infarctions (2). Heparin acts on thrombin, which then inactivates proteins in the coagulation pathway (3). Heparin is not a single molecule but rather describes a family of glycosaminoglycans (GAGs) with specific polysaccharide components of varying length and sulfation. The most common formulations in clinical use are unfractionated heparin and low molecular weight heparin. The molecular mass of unfractionated heparin is typically between 5 and 30 kDa, whereas low molecular weight heparin is usually 1–8 kDa (1). Endogenously, heparan sulfate, structurally related to heparin, is intrinsic to the cell migration properties of chemokines, a family of ~50 structurally homologous chemotactic cytokines.

Chemokines induce the migration of cells by activating G protein-coupled receptors. The direction of chemotaxis is...
encoded in a concentration gradient; secreted chemokines are immobilized, by the heparan sulfate GAG chains of proteoglycans, along the endothelial surface. This establishes a gradient with the highest concentrations closest to the origin of secretion (4–6). Originally recognized as the major promoter of leukocyte trafficking, chemokines have been found to participate in a variety of developmental and housekeeping roles, including lymphopoiesis, myelopoiesis, embryogenesis (7), angiogenesis (8), cardiogenesis (9), neuron migration, and cerebral development (10, 11).

Following myocardial ischemia/reperfusion injury, there is increased expression of the chemokine CXCL12 (SDF-1) in cardiac myocytes, endothelial cells, smooth muscle cells, and fibroblasts (12–14). CXCL12 possesses cardioprotective properties when administered either pre- or post-ischemia including reduced infarct zone, decreased scar tissue, increased angiogenesis, resistance to hypoxia/reoxygenation damage, and improved cardiac function (12–19). Despite functional analyses in vitro and in vivo, the mechanism of CXCL12-induced cardioprotection is disputed: CXCL12 may act through direct activation of growth and survival signaling pathways in cardiomyocytes (12, 13) and/or enhance cardiac regeneration by recruiting hematopoietic stem and endothelial progenitor cells into the heart (18, 20–24).

Although there is ambiguity regarding the cardioprotective mechanism, researchers are aggressively pursuing therapies as evidenced by numerous methods for chemokine administration to ischemic tissue. These include: CXCL12 attached to poly(ethylene glycol)-fibrin patches (18), intravenously introducing self-assembling nanofibers with a timed chemokine release mechanism (15), and injection of viruses engineered to produce protein (21). In each case, CXCL12 treatment is effective, but variations in protection may be related to stability, dosage, or drug interactions. We recently demonstrated that CXCL12 is cardioprotective over a narrow concentration range, whereas a constitutively dimeric form of the chemokine exerts little to no effect (19). Because heparin is commonly administered in response to myocardial infarctions (2) and induces CXCL12 dimerization (25, 26), we hypothesized that the protective properties of CXCL12 may be attenuated by interactions with soluble heparin in a modern clinical setting.

The vital role of GAG binding in CXCL12-directed migration has motivated a series of studies, but its impact on chemokine activity, stability, and receptor interactions remains poorly defined. CXCL12 recognition by heparin oligosaccharides has been characterized using mutagenesis, in vitro binding assays, NMR, crystallography, and molecular modeling (27–31). We showed previously that heparin disaccharides bind preferentially to the CXCL12 dimer (26), and others have proposed a binding mode localized to the dimer interface (27, 28). Consequently, we speculated that mutations previously used to define the binding site might reduce heparin affinity indirectly by limiting dimerization.

Using the previously described preferentially monomeric (CXCL12_{H25R}) and constitutively dimeric (CXCL12_{2}) variants, we examined the affinity of distinct CXCL12 oligomers for heparin oligosaccharides of varying length. To deconvolute chemical shift perturbations caused by heparin binding from those reporting on dimerization, we monitored oligosaccharide binding to the CXCL12_{2} dimer by two-dimensional NMR. Heparin-induced chemical shift changes and mutagenesis of basic side chains was used to further refine the GAG binding interface. Binding studies with a CXCR4 N-terminal peptide demonstrate partial overlap and competition with the heparin interface. In contrast to previous reports that heparin binding to Lys-1 protects CXCL12 from cleavage by dipeptidyl peptidase-IV (DPPIV/CD26), NMR, surface plasmon resonance (SPR), and enzymatic assays demonstrate that reduced cleavage rates are a result of heparin-induced dimerization rather than direct association with the N terminus.

Our results suggest that heparin blocks the cardioprotective effect of CXCL12 by promoting dimerization. Heparin binds a site orthogonal to the dimer interface that partially overlaps the CXCR4 N terminus binding site and does not involve Lys-1. In principle, CXCL12 cardioprotection may be enhanced by modifications that alter its ability to self-associate or interact with heparin.

**EXPERIMENTAL PROCEDURES**

**Protein Engineering, Expression, and Purification—**CXCL12, CXCL12_{H25R}, and CXCL12_{2} were expressed and purified as previously described (26, 32). All other chemokine variants were produced via mutagenesis of CXCL12 and CXCL12_{2} constructs with complementary primers and the QuikChange site-directed mutagenesis kit (Stratagene) per the manufacturer’s instructions. All expression vector inserts were confirmed by DNA sequencing.

**Ex Vivo Cardioprotection Assay—**Isolated hearts were perfused for 15 min, followed by aerobic perfusion 15 min prior to 30 min of global, no-flow ischemia, and 180 min of reperfusion. Aerobic perfusion including either 50 nM CXCL12, 50 units ml⁻¹ heparin, or 50 nM CXCL12 plus 50 units ml⁻¹ heparin. Hearts subjected to the above mentioned perfusion sequence in the absence of both CXCL12 and heparin served as ischemic controls. Hearts perfused continuously for 245 min served as nonischemic controls. Administration of the perfusion sequence, monitoring of cardiac function, and analysis of infarcted tissue was performed as previously described (19, 33, 34). Each dosage group consisted of four replicates. Resistance to injury from myocardial infarction/reperfusion was determined by a reduction in infarct size and/or an increase in recovery of developed pressure.

**NMR Spectroscopy—**NMR experiments were performed on a Bruker DRX 600 instrument equipped with a ¹H, ¹⁵N, ¹³C TXI cryoprobe. Titration experiments were performed with either 50 µM [¹H,¹⁵N]glymetCXCL12 or [¹H,¹⁵N]glymetCXCL12 in 25 mM D-MES buffer (pH 6.8), 200 mM NaCl, 10% (v/v) D₂O, and 0.02% (w/v) NaN₃. CXCL12 and CXCL12_{2} H² assignments were transferred from previously published chemical shift tables (Biological Magnetic Resonance Bank identification code 16145 and 15633, respectively). Chemical shift perturbations were tracked using CARA (35). HCCCH-TOCSY spectra were collected on 0.4 mM [¹H,¹⁵N,¹³C]CXCL12_{2}, 25 mM D-MES buffer (pH 6.8), 200 mM NaCl, 10% (v/v) D₂O, and 0.02% (w/v) NaN₃ in the presence of 0.0, 0.3, and 0.6 mM heparin desaccharide of degree of polymerization (dp)10. Partial CXCL12_{2}
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side chain assignments were transferred from previously published chemical shift table (Biological Magnetic Resonance Bank identification code 15633), and complete assignments were achieved with three-dimensional HCCH-TOCSY and three-dimensional $^{15}$N-edited NOESY-HSQC. All of the experiments were performed at 298 K.

Binding site competition between CXCR4$_{1-38}$ and tetrascarhide dp4 was assessed using $^{15}$N HSQC experiments at 298 K. 200 nM CXCR4$_{1-38}$ was added to a sample comprised of 50 nM [U-$^{15}$N]CXCL12$_2$, 150 nM dp4, 25 mM d-MES, 200 mM NaCl, 10% (v/v) D$_2$O, and 0.2% (v/v) NaN$_3$.

Disaccharide and tetrascarhide induced dimerization of CXCL12 were assessed using $^{15}$N HSQC experiments at 298 K. A sample containing 10 nM [U-$^{15}$N,$^{13}$C]CXCL12, 25 mM d-MES (pH 6.8), 10% (v/v) D$_2$O, and 0.2% (v/v) NaN$_3$ was titrated with dp2 or dp4 (0, 5, 10, 20, and 30 μM).

Preparation of Heparin Biochip and SPR Analysis—The biotinylated heparin was immobilized to a streptavidin chip based on the manufacturer’s protocol. The successful immobilization of biotinylated heparin was confirmed by the observation of a 250-resonance unit increase in the sensor chip. SPR measurements were performed on a BIACore 3000 (GE Healthcare). The buffers were filtered and degassed prior to analysis. The protein samples were diluted in HBS-EP buffer (0.1 M HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4). Different dilutions of protein samples were injected at a flow rate of 30 μl min$^{-1}$. At the end of the sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation. After a 3-min dissociation time, the sensor surface was regenerated by injecting 30 μl of 2 M NaCl. For competition with immobilized heparin, protein (50 nM) was mixed with certain concentrations of low molecular mass heparin (dp$_{avg}$20), heparin oligosaccharides (di (dp2), tetra (dp4), hexa (dp6), etc.) in HBS-EP buffer and injected over the heparin chip at a flow rate of 30 μl min$^{-1}$. For receptor competition studies, CXCL12$_2$ (50 nM) was premixed with CXCR4$_{1-38}$ (500 nM) and injected over the heparin chip. The response was monitored as a function of time at 298 K.

Fluorescence Polarization Assay—Fluorescence polarization assays were performed on a fluorometer (PTI) equipped with automated polarization systems. The intensity of CXCL12(3–68) was normalized to [U-$^{15}$N]CXCL12(3–68) internal standard. The normalized intensity at each time point was subtracted from 5, the maximum intensity of unlabeled/labeled CXCL12(3–68), and then the half-life was calculated using nonlinear regression (pro Fit; QuantumSoft).

RESULTS

Heparin Attenuates the Cardioprotective Effect of CXCL12—CXCL12 is a potential anti-ischemic drug that has been observed to decrease the amount of infarcted tissue and improve cardiac function when administered either pre- or post-myocardial infarction (12–14, 16, 18). We previously showed that CXCL12 cardioprotection is mediated by the monomeric species (19). The antithrombotic properties of heparin are well established and highlighted by its presence as a clinical mainstay for the treatment of myocardial infarction. Because soluble heparin binds to and promotes dimerization of the chemokine (25, 26) and inhibits cell migration in vitro (30), we hypothesized that this interaction would also attenuate CXCL12-mediated cardioprotection.

Using an isolated rat heart model of ischemia/reperfusion injury, the cardioprotective effect of CXCL12 was measured in the presence of medicinal heparin (average molecular mass = 14 kDa; dp$_{avg}$42) (36). Increased resistance to injury from myocardial ischemia/reperfusion was determined by a reduction in infarct size and/or increase in recovery of left ventricle diastolic pressure. Whereas CXCL12 (50 nM) significantly reduced both measures of ischemia/reperfusion injury in isolated buffer-perfused rat heart relative to untreated controls, inclusion of heparin sodium (50 units ml$^{-1}$) completely eliminated the cardioprotective effect of CXCL12 (Fig. 1). This suggests that in a clinical setting, co-administration of heparin could attenuate the therapeutic benefit of CXCL12, underscoring the value of the molecular details for this chemokine/GAG interaction.
Heparin Dodecasaccharide Produces Substantial Line Broadening in CXCL12—Heparin-insensitive CXCL12 variants were identified by mutagenesis of the BBXB motif (residues 24–27) more than 10 years ago. Nonetheless, structural data on the binding interface is limited to a crystal structure of a heparin disaccharide bound to CXCL12 and NMR chemical shift data for a disaccharide and octasaccharide (30, 31). We monitored the binding of longer chain heparin oligosaccharides using two-dimensional $^1$H–$^15$N HSQC NMR spectroscopy. Residues that exhibit large relative changes in chemical shift correlate with significant involvement in heparin binding—identifying the interface. Titration of 50 μM CXCL12 with increasing concentrations of heparin dodecasaccharide (0–150 μM) produced chemical shift changes and line broadening over a large area of the molecule including substantial perturbations clustered at the dimer interface (Fig. 2). Because heparin binding promotes CXCL12 dimerization (25, 26), we concluded that heparin-induced shifts arose from a combination of GAG-protein and protein–protein contacts.

High Affinity GAG Binding Requires CXCL12 Dimerization and Is Chain Length-dependent—To simplify analysis of the binding interaction by removing the monomer-dimer equilibrium, we tested whether the covalent dimer CXCL12$_{2}$ or preferential monomer CXCL12$_{24–27}^{125SR}$ are appropriate models for CXCL12–GAG binding (26, 32). SPR was used to measure the interaction of immobilized porcine intestinal heparin (average molecular mass = 14 kDa; $dp_{av} = 42$) with CXCL12, CXCL12$_{24–27}^{125SR}$ and CXCL12$_{2}$ (Table 1). Our results indicate that both monomeric and dimeric CXCL12 bind heparin but exhibit different rates of association and dissociation. Not surprisingly, dimeric CXCL12$_{2}$ binding is 200 times stronger than monomeric CXCL12$_{24–27}^{125SR}$. Wild type CXCL12 binds with an intermediate affinity that presumably reflects the relative contributions of monomers and dimers in equilibrium.

Next, we used SPR to measure the ability of immobilized heparin to displace CXCL12 from soluble heparin fragments of varying length. CXCL12 variants (50 nM) and various oligosaccharides (dp2 to dp20) were premixed and presented to immobilized heparin (average molecular mass = 14 kDa; $dp_{av} = 42$). Supplemental Fig. S1 illustrates the changes in response units as a function of heparin concentrations that were used to calculate the IC$_{50}$ and $K_d$ values (Table 2). For CXCL12 and CXCL12$_{24–27}^{125SR}$ little or no displacement occurred for complexes with dp2, dp4, dp6, and dp8 heparin, but dp10 and longer oligosaccharides reduced binding to the immobilized heparin. The dp20 produced a sharp change in CXCL12 and CXCL12$_{24–27}^{125SR}$ response units characteristic of a change in complex stoichiometry. This is consistent with longer heparin chains increasingly promoting dimerization (25). It is also possible that dp20 may induce higher order oligomerization. The similar response of CXCL12$_{24–27}^{125SR}$ to wild type is consistent with its 10-fold weaker dimerization affinity (26). In contrast to CXCL12 and CXCL12$_{24–27}^{125SR}$, the affinity of CXCL12$_{2}$ for heparin increases in direct proportion to oligosaccharide length. CXCL12$_{2}$ had the tightest binding affinity and its association with the immobilized heparin was reduced the greatest by soluble heparin, regardless of size.
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Based on SPR results, we speculated that NMR measurements of CXCL12 binding to purified heparin oligosaccharides of varying length could provide useful structural details. To eliminate contributions from CXCL12 self-association and define chemical shift perturbations unambiguously caused by heparin binding, we used the constitutively dimeric CXCL12 variant. The titrations of various, heparin oligosaccharides of defined length (dp2, 4, 6, 8, 10, 12, 16, and 18) into CXCL12 were monitored by two-dimensional 1H-15N HSQC NMR experiments (supplemental Figs. S2–S4). As illustrated in Fig. 3B, the heparin tetrasaccharide (dp4) produced perturbations confined mainly to residues 23–30 (β1 strand), 35–43 (β2 strand), and 47–50 (β3 strand). Titration of heparin octasaccharide (dp8) induced corresponding shifts and additionally included residues 17–19 in the N-loop (Fig. 3C). Increasing the oligosaccharide length resulted in line broadening across the 1H-15N HSQC signals for arginine (Fig. 3D). Analysis of our NMR titration data suggests a key role for H-1 and H-327 residues in direct heparin binding. As illustrated in supplemental Fig. S6, the function of each variant was confirmed via measurement of the calcium flux response. Mutations in the CXCL12 background report only on heparin binding, whereas the affinity of CXCL12 background mutants reflects both direct and indirect effects. The binding affinity of each mutant for immobilized heparin (average molecular mass = 14 kDa; dpavg,42) was determined by SPR (Table 3). Neither the single mutants nor the K24S/K27S variant reduced the CXCL12 binding affinity by more than 2-fold. This stands in stark contrast to the >1,000-fold change in affinity the mutations produce in the wild type CXCL12 background and underscores the importance of dimerization in heparin binding.

Analysis of our NMR titration data suggests a key role for Lys-24, Arg-41, and Arg-47 in heparin binding. Mutagenesis studies were undertaken to measure the contributions of these residues in direct heparin binding by producing alanine mutants in both the CXCL12 and CXCL12 background. As illustrated in supplemental Fig. S6, the function of each variant was confirmed via measurement of the calcium flux response. Mutations in the CXCL12 background report only on heparin binding, whereas the affinity of CXCL12 background variants reflects both direct and indirect effects. The binding affinity of each mutant for immobilized heparin (average molecular mass = 14 kDa; dpavg,42) was determined by SPR (Table 3). In the CXCL12 background, single or multiple amino acid substitutions produce substantial changes in affinity. In contrast, single and double residue substitutions have little effect on CXCL12 binding. Mutagenesis of all three basic residues is necessary to significantly reduce heparin binding. These results

| Compounds | IC50 | Ki |
|-----------|------|----|
|         | CXCL12 | CXCL12 | CXCL12 |
|         |       |       |       |
| dp2      | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp4      | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp6      | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp8      | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp10     | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp12     | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp16     | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| dp20     | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |
| Heptanese | >10 μM | >3.8 μM | >10 μM | >3.8 μM | >10 μM | >3.8 μM |

The side chains of Arg-8 and Arg-12 display a mixture of perturbed and broadened resonances; the broadened H-2, H-6, and H-8 resonances and perturbed H-8 suggest a nominal contribution by the guanidinium groups. Lys-54, Lys-56, and Lys-64 had varying degrees of incomplete broadening in H-4, H-6, H-17, and H-18 with no perturbation in H-10, suggesting no direct role in heparin binding through the primary amine. Consistent with the Lys-68 H-1 resonance in the presence of all oligosaccharides tested, the side chain nuclei do not respond to decasaccharide and appear to be uninvolved in heparin binding.
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FIGURE 3. Heparin binds orthogonal to the CXCL12 dimer interface in a size-dependent manner. A, CXCL12 ribbon and surface representation in the absence of heparin; the two protomers are shaded in light and dark gray (N-terminal residues 1–8 are absent for ease of viewing). B–D, CXCL12 surface illustration in the presence of 3 equivalents of dp4 (B), dp8 (C), and dp12 (D). Residues that experience substantial chemical shift perturbations (magenta) and complete line-broadening (green) are mapped onto the CXCL12 NMR structure (Protein Data Bank code ID 2K01). E and F, HCCH-TOCSY H- strips of all CXCL12, arginine (E) and lysine (F) residues in the absence and presence of heparin decasaccharide. Side chain correlations are labeled, and a dashed line indicates the positions of apo-CXCL12 resonances. Strong resonances near the Arg-47 H- peak in the presence of heparin are signals from Arg-8 that bleed through from a neighboring plane of the three-dimensional spectrum.

are consistent with a model in which clusters of positively charged side chains in two CXCL12 monomers join to form an elongated heparin-binding site that crosses the six-stranded β sheet of a CXCL12 dimer.

The Heparin Interface Partially Overlaps the CXCR4 N-terminal Binding Site—CXCR4-mediated cell migration is presumed to require that GAG-immobilized CXCL12 oligomers in the extracellular matrix be presented to the receptor, which transmits the external migratory signal to the internal cellular machinery. The initial interaction with CXCR4 is mediated through the receptor N terminus; however, inconsistencies exist as to whether this binding site overlaps with that of heparin (28, 30). The heparin-binding surface defined by chemical shift mapping is shared with the receptor-binding surface defined in structural studies with CXCR4 N-terminal peptides, including key sulfotyrosine binding residues Arg-47 and Lys-27 (32, 37). We tested the ability of an unsulfated CXCR4 N-terminal peptide comprising the first 38 amino acids, CXCR41–38, to either displace dp4 or form a ternary complex by binding simultaneously to a complementary site. Addition of CXCR41–38 had essentially no effect on the HSQC spectrum of dp4-bound CXCL12 (Fig. 4, A and B), demonstrating that heparin competes effectively with CXCR4 for CXCL12 binding. Using SPR, we tested whether the large chain immobilized heparin oligosaccharides (average molecular mass = 14 kDa; dpavg42) could compete CXCL12 from CXCR41–38. As illustrated in Fig. 4C, CXCL12 (50 nM) preincubated with CXCR41–38 (500 nM) was unable to bind the immobilized heparin despite a 1000-fold higher affinity for the heparin.

Heparin Indirectly Protects the N Terminus from CD26/DPPIV Degradation—DPPIV/CD26 is a well known exopeptidase found in both a membrane-bound and a soluble form. It recognizes CXCL12 and Cleaves between residues 2 and 3 to produce a truncated variant with a reduced affinity for, and an inability to activate, CXCR4 (38). Sadir et al. (29) used Western blot analysis to demonstrate that heparin molecules of increasing chain length protected CXCL12 from DPPIV/CD26-mediated degradation and attributed this effect to the direct association of heparin with Lys-1. To detect the participation of Lys-1 in the CXCL12/heparin interaction, we monitored dp10-induced chemical shift perturbations by HCCH-TOCSY. Although

| TABLE 3 | Summary of SPR kinetic data from the interaction of CXCL12 variants with heparin (average molecular mass = 14 kDa; dpavg42) |
|---------|---------------------------------------------------------------|
| $$k_{on}$$ | $$k_{off}$$ | $$K_d$$ |
| CXCL12 variants | | | |
| CXCL12 | 7.6 x 10^5 | 3.8 x 10^4 | 7.9 x 10^8 |
| K27A | 4.1 x 10^5 | 2.2 x 10^4 | 5.4 x 10^8 |
| R41A | 8.6 x 10^5 | 1.9 x 10^4 | 2.2 x 10^8 |
| R47A | 2.7 x 10^5 | 1.9 x 10^4 | 7.0 x 10^9 |
| K27A/R41A | 1.6 x 10^5 | 1.2 x 10^4 | 7.5 x 10^9 |
| K27A/R4A | 5.7 x 10^5 | 3.3 x 10^4 | 5.8 x 10^8 |
| K41A/R47A | 9.0 x 10^5 | 3.0 x 10^4 | 3.3 x 10^8 |
| K27A/R41A/R47A | 5.2 x 10^5 | 3.7 x 10^4 | 7.1 x 10^8 |
| K24S | 2.2 x 10^5 | 2.8 x 10^4 | 1.3 x 10^8 |
| K27S | 6.2 x 10^5 | 2.1 x 10^4 | 3.4 x 10^9 |
| K24S/K27S | 7.0 x 10^5 | 7.2 x 10^4 | 1.0 x 10^8 |
| Δ1–2 | 1.1 x 10^4 | 4.5 x 10^4 | 4.1 x 10^9 |
| CXCL12 variants | | | |
| CXCL12 | 4.9 x 10^10 | 1.5 x 10^-3 | 3.1 x 10^-6 |
| K27A | 2.4 x 10^10 | 2.1 x 10^-1 | 8.8 x 10^-7 |
| R41A | 7.8 x 10^10 | 8.2 x 10^-2 | 1.1 x 10^-5 |
| R47A | 2.0 x 10^10 | 1.5 x 10^-2 | 7.5 x 10^-8 |
| K27A/R41A | 6.1 x 10^10 | 8.0 x 10^-4 | 1.3 x 10^-6 |
| K27A/R47A | 1.5 x 10^10 | 3.8 x 10^-3 | 2.5 x 10^-5 |
| R41A/R47A | 2.0 x 10^10 | 3.2 x 10^-3 | 1.6 x 10^-5 |
| K27A/R41A/R47A | 8.4 x 10^10 | 3.4 x 10^-2 | 4.0 x 10^-5 |
| K24S | 2.4 x 10^10 | 2.2 x 10^-1 | 9.9 x 10^-6 |
| K27S | 2.7 x 10^10 | 1.1 x 10^-1 | 4.1 x 10^-4 |
| K24S/K27S | 2.2 x 10^10 | 4.4 x 10^-3 | 2.0 x 10^-5 |
| Δ1–2 | 2.5 x 10^10 | 2.4 x 10^-2 | 9.6 x 10^-8 |
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We recently demonstrated that the half-life of the CXCL12-H9262 (39), and therefore hypothesized that heparin may indirectly protect Lys-1 by promoting CXCL12 dimerization. We incubated 10 μM CXCL12 and 0.2 ng μl⁻¹ DPPIV/CD26 alone or in the presence of dp2 or dp4 heparin. The reaction was monitored by following CXCL12(3–68) half-life of 26.5 ± 8.7 min (39), herein the buffer control exhibited a half-life of 41.5 ± 10 min. As shown in Fig. 6, protection of CXCL12 N-terminal cleavage is dependent on the size of heparin. Whereas dp2 does not provide protection (τ₁/₂ = 17.1 ± 2.4 min), dp4 slows the half-life to 1257 ± 75 min. The long term stability of DPPIV/CD26 was confirmed by measuring the half-life of CXCL12 degradation after 4 days (data not shown). The drastic difference in heparin-promoted dimerization between dp2 and dp4 is consistent with previous mass spectrometry studies at similar protein and oligosaccharide concentrations (25). We collected two-dimensional HSQC spectra at CXCL12 and heparin concentrations (25). We collected two-dimensional HSQC spectra at CXCL12 and heparin concentrations (25).

**DISCUSSION**

Heparin is a well established antithrombotic agent and a clinical mainstay for the treatment of myocardial infarction. More recently, the anti-ischemic effects of CXCL12 have led to several novel treatment strategies. CXCL12 binds to soluble heparin and extracellular matrix GAGs. In the present study, we monitored the interaction of CXCL12 with soluble heparin by several methods and defined a binding site that may also be relevant to immobilization in the extracellular matrix. Although interactions with cell surface GAGs are essential for...
chemotactic cell migration, soluble GAGs can block chemokine activity. We hypothesized that simultaneous administration of chemokine and heparin would diminish the protective properties of CXCL12 rather than synergizing their individual beneficial effects. As predicted, co-administration inhibited the cardioprotective effect of CXCL12 in an isolated rat heart model. This model reports directly on the function of cardiac tissue. If CXCL12 on heparin activity is completely unknown and warrants future exploration.

The interaction between CXCL12 and heparin has been the focus of five papers over the last 13 years. The original mutagenic characterization by Amara et al. (28) identified residues Lys-24, His-25, and Lys-27 at the dimer interface as a canonical BXXB heparin-binding motif that is distinct from the CXCR4 N-terminal binding site. In 2001, Arg-41 and Arg-43 were proposed as additional contributors to the heparin binding interface. In the proposed structural model, heparin bound along the CXCL12 dimer interface, primarily making contacts with the β1-strand of each protomer (27). Later, a crystal structure of CXCL12 in complex with a heparin disaccharide analog identified two distinct binding sites: one high affinity site at the dimer interface and a second site between the N-loop region and the C-terminal helix (30). Finally, Sadir et al. (29) further expanded the proposed binding site to include the N termini; they concluded that the direct interaction of Lys-1 with heparin was responsible for limited proteolysis by DPPIV/CD26. Despite the wealth of data published on this crucial interaction, there are several inconsistencies in the predicted binding mode.

**CXCL12/Heparin Interface**—The monomer-dimer equilibrium and lack of data using long chain heparin molecules has significantly hindered the structural characterization of CXCL12-heparin complexes. We used the CXCL12-dimer to eliminate complications from CXCL12 self-association and monitor interactions with isolated heparin molecules ranging in size from dp2 to dp20. Whereas titration of heparin dodecasaccharide into wild type CXCL12 produced substantial line broadening throughout the dimer interface, CXCL12 enabled visualization of heparin-specific contacts. Our results differ from previous structural models. Instead of an interface mostly comprising the β1 strands and N terminus of each protomer in a dimer (Fig. 7A) (27, 29, 31), our data suggest that heparin drives dimer formation by interacting nearly orthogonal to the dimerization interface. We propose that the interface between CXCL12 and heparin crosses the entire six-stranded β-sheet, does not involve the N terminus, and is primarily mediated by Arg-20, Lys-24, Lys-27, Arg-41, Lys-43, and Arg-47 with the potential peripheral involvement of Arg-8 and Arg-12 (Fig. 7B).

A recently published model illustrated the octasaccharide-binding site more or less along the dimer interface; the terminal sugars interact with Arg-8 and Arg-12 on each protomer, whereas the core saccharides contact Lys-24, His-25, Lys-27, and Arg-41 (31). Although both Arg-8 and Arg-12 exhibit modest 1H, 15N chemical shift perturbations and broadening of H10, H9, and H8 resonances, the H8 spins are minimally perturbed and indicate little role for the guanidinyl groups in heparin binding. These results are consistent with previous R8E/R12E mutations that actually increased CXCL12 affinity for low molecular weight heparin (30). Perhaps removal of Arg-8 and Arg-12 enhances the interaction between heparin and basic residues in the chemokine cleft formed by the N-loop and β3 strand.

It should also be noted that the biological role for chemokine/GAG interactions is varied and context-dependent. Although there is a possibility that heparin and heparan sulfate recognize distinct binding sites, the similarity of their oligosaccharide structures and the inhibitory effect of heparin in vivo suggest that they share a single, overlapped binding site. At the same time, there may be important differences in the contributions of individual amino acid or glycan residues to the interaction. Our studies have identified residues that are likely to be involved in binding endogenous GAGs, but the unique structural features of heparin (i.e., greater charge density compared with heparan sulfate and other extracellular matrix GAGs) may lead to slight differences in their binding modes.

**Heparin and CXCR4 Sites Overlap**—Previous analyses disagree as to whether the heparin and CXCR4 binding sites overlap (28, 30). Using a N-terminal peptide of CXCR4, we directly demonstrate competition between the receptor and heparin. The inability of the CXCR4 receptor peptide to dissociate dp4 does not prove that a native N terminus would also be insufficient. The CXCR4 N terminus can be O-sulfated at three tyrosine residues that together increase complex affinity from 4.5 ± 2.2 to 0.2 ± 0.2 μM (40). However, our results suggest that some of the surfaces occupied by CXCR4 sulfotyrosines may also be utilized for recognition of the sulfate moieties decorating GAG molecules. Considering the structural similarity between heparin and endogenous heparan sulfate, we speculate that similar competition occurs in vivo.

**CXCL12 N Terminus Does Not Bind Heparin**—The initial characterization of GAG binding excluded a role for Lys-1, as an antibody specific to the Lys-1–Pro-2 dipeptide was able to recognize CXCL12 bound to cell surface heparin (28). However, the same antibody was later used to show that heparin...
molecules equal to and larger than dp12 compete for the N terminus (29). Our measurements of side chain chemical shift perturbations and SPR analysis of CXCL12(3–68) and CXCL122(3–68) clearly demonstrate that Lys-1 plays no role in binding to heparin oligosaccharides. Consequently, we concluded that heparin-mediated protection of the CXCL12 N terminus from proteolysis must be an indirect effect related to the GAG-induced oligomerization.

Dimerization Inhibits CXCL12 Degradation—DPPIV/CD26 is a multidomain enzyme that possesses distinct recognition and catalytic regions (41). To study the enhanced serum stability of CXCL122 compared with CXCL12, we recently monitored in vitro degradation by DPPIV/CD26 (39). The half-life of dimeric CXCL12 is 25-fold greater than wild type (39). Because oligosaccharide-induced dimerization is chain length-dependent, we hypothesized that heparin may possess a similar size-mediated influence on protection of DPPIV/CD26 degradation. Whereas incubation of CXCL12 with dp2 had no effect on degradation, dp4 delayed N-terminal cleavage nearly 30-fold. The lack of structural data for DPPIV/CD26 in complex with protein substrates makes the mechanism of enhanced stability unclear. The simplest explanation is the potential for overlap of the DPPIV/CD26 binding site and the CXCL12 dimerization interface. Most DPPIV/CD26 substrates are compact peptides with molecular masses less than 10 kDa (42). The large, dimeric CXCL12 may therefore also prevent the dimerization of DPPIV/CD26, which has been shown to reduce $k_{cat}$ 30-fold (43). Matrix metalloprotease-2 also degrades CXCL122 slower than CXCL12 (39). Together, this suggests that heparin may indirectly protect CXCL12 from numerous other enzymes such as matrix metalloproteases 1, 3, 9, 13, and 14 (44), cathepsin G (45), and elastase (46). Because of growing clinical attention, it will be of interest to identify CXCL12 variants that maximize its cardioprotective properties while minimizing secondary interactions.

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