CXCR3 and Heparin Binding Sites of the Chemokine IP-10 (CXCL10)*

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Gabriele S. V. Campanella, Elizabeth M. J. Lee, Jieti Sun, and Andrew D. Luster‡
From the Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

The chemokine IP-10 (interferon-inducible protein of 10 kDa, CXCL10) binds the G protein-coupled receptor CXCR3, which is found mainly on activated T cells and NK cells, and plays an important role in Th1-type inflammatory diseases. IP-10 also binds to glycosaminoglycans (GAGs), an interaction thought to be important for its sequestration on endothelial and other cells. In this study, we performed an extensive mutational analysis to identify the CXCR3 and heparin binding sites of murine IP-10. The mutants were characterized for heparin binding, CXCR3 binding, and the ability to induce chemotaxis, Ca2⁺ flux, and CXCR3 internalization. Double mutations neutralizing adjacent basic residues at the C terminus did not lead to a significant reduction in heparin binding, indicating that the main heparin binding site is probably located along the C-terminal helix. Alanine exchange of Arg-22 had the largest effect on heparin binding, with residues Arg-20, Ile-24, Lys-26, Lys-46, and Lys-47 further contributing to heparin binding. A charge change mutation of Arg-22 resulted in further reduction in heparin binding. The N-terminal residue Arg-8, preceding the first cysteine, was critical for CXCR3 signaling. Mutations of charged and uncharged residues in the loop regions of residues 20–24 and 46–47, which caused reduced heparin binding, also resulted in reduced CXCR3 binding and signaling. CXCR3 expressing GAG-deficient Chinese hamster ovary cells revealed that GAG binding was not required for IP-10 binding and signaling through CXCR3, which suggests that the CXCR3 and heparin binding sites of IP-10 are partially overlapping.

Interferon-inducible protein, 10 kDa (IP-10) belongs to the superfamily of chemokines (chemotactant cytokines), that are involved in the activation and recruitment of leukocytes, as well as non-hematopoietic cells (1). Chemokines are 8–10-kDa proteins that have been subdivided based on the first two cysteine residues into four subfamilies. IP-10 is a member of the CXC subfamily and was first identified as a gene markedly induced by interferon γ (2) and has since been shown to be a potent chemotactant of activated T cells (3). It is expressed constitutively at low levels in thymic, splenic, and lymph node stroma (4), but its expression can be highly induced by interferon α, β, and γ, and LPS in a variety of cell types, including endothelial cells, keratinocytes, fibroblasts, mesangial cells, astrocytes, monocytes, and neutrophils (5). It has been demonstrated to be highly expressed in many Th1-type inflammatory diseases, including skin diseases (6–8), atherosclerosis (9), multiple sclerosis (10, 11), allograft rejection (12, 13), and others. Studies with inhibitory antibodies and IP-10-deficient mice have revealed that IP-10 plays an important role in the recruitment of effector T cells into inflammatory tissues (14–17). IP-10 has also been shown to have angiostatic (18–21) and antitumor activity (22, 23).

Chemokines activate leukocytes by binding to 7 transmembrane, G protein-coupled receptors. IP-10 binds to the CXC chemokine receptor 3 (CXCR3), which it shares with two other ligands, interferon-inducible T cell-α chemotactant (I-TAC/CXCL11) and monokine induced by γ-interferon (Mig/CXCL9). IP-10, like many chemokines, also binds to cell surface glycosaminoglycans (GAGs) (21, 24). While our understanding of the biological activities of the CXCR3 ligands has increased, relatively little is known about the importance of their interaction with GAGs. It has been postulated that GAGs on the cell bearing the 7 transmembrane receptors facilitate chemokine binding to their high affinity receptor (25), while GAGs on endothelial cells and in the extracellular matrix might be important for retaining chemokines close to their site of secretion (26). Furthermore, it has been suggested that GAGs might also be involved in the signaling of chemokines, as has been recently shown for RANTES (CCL5) (27) and PF4 (platelet factor 4, CXCL4) (28). GAGs have also been shown to be important for the anti-HIV effect of SDF-1α (stromal cell-derived factor-1α, CXCL12) (29) and RANTES (30, 31). In addition, GAGs might also be important for chemokine activity on non-hematopoietic cells, such as endothelial cells.

In this study, we performed an extensive mutational study to locate the CXCR3 and heparin binding sites of murine IP-10 to begin to dissect the role of CXCR3 and GAG binding in the biological activity of IP-10. We found that the N-terminal residue Arg-8 as well as residues in the loop regions 22–26 and 46–47 are important for CXCR3 binding. Interestingly, these same loop regions are also the major heparin binding site, suggesting that the CXCR3 and heparin binding sites of IP-10 are partially overlapping.

EXPERIMENTAL PROCEDURES

Materials—Recombinant murine IP-10 was obtained from Peprotech (Rocky Hill, NJ), [125I]-labeled IP-10 (human) was purchased from...
PerkinElmer Life Sciences. Endotoxin testing reagents were from Charles River Laboratories, Wilmington, MA. BCA reagents were from Pierce. All other materials were of biological grade and purchased either from Sigma or Fisher.

**Cell Culture and Transfection**—300–19 cells were maintained in complete RPMI, 10% FCS. 300–19 cells transfected with human CXCR3 (30–19/mCXCR3) were a gift from S. Tonegawa. CHO cells and CHO/mCXCR3 were a gift from B. Moser. CHO K1 cells and CHO/mCXCR3 cells were maintained in high glucose medium supplemented with 10% FCS and 1% low endotoxin BSA. Cells were washed into the same buffer at a concentration of 5 × 10^5 cells/ml in calcium flux analysis. Cells were washed and resuspended at 5 × 10^6 cells/ml in calcium flux buffer supplemented with 0.5 M NaCl. Radioactivity was counted after addition of scintillation fluid in a Microbeta counter (Wallac). The data were analyzed with GraFit (34). Each experiment was performed in duplicate and repeated a minimum of two times.

**Calcium Mobilization**—Internalization of mCXCR3 was measured by a continuous monitoring of the peak fluorescence at 340/380 nm. Each experiment was repeated at least twice. The fluorescence intensity emitted at 510 nm in response to sequential excitation at 340 and 380 nm was analyzed with a Photomultiplier (Random Access Monochromator) fluorimeter (Photon Technology International, Monmouth Junction, NJ). The data were recorded as excitation fluorescence intensity emitted at 510 nm in response to sequential excitation at 340 and 380 nm and are presented as the relative ratio of fluorescence at 340/380 nm. Fold induction (FI) of each stimulation was calculated by dividing the peak fluorescence ratio after stimulation of fluorescence at 340/380 nm and are presented as the relative ratio of fluorescence at 340/380 nm.

**CXCR3 and Heparin Binding Sites of IP-10**

Amino acid sequence of IP-10 and selected CXC chemokines. Residues in murine (m) IP-10 conserved in relationship to human (h) IP-10 are shown in bold. Residues in IL-8 involved in receptor binding are double underlined (36, 37, 49), and residues of IL-8 and PF4 implicated in heparin binding are single underlined (42, 43).

**CXCR3 and Heparin Binding Sites of IP-10**

**RESULTS**

**Mutagenesis and Purification of IP-10 Mutants**—Residues of mIP-10 were chosen for mutagenesis by homology to the human and murine CXCR3 ligands IP-10, Mig, and IP-10 protein were loaded on a 1 ml Heparin HiTrap or S-Sepharose FF (cationic exchange) column (both from Amersham Biosciences) equilibrated in 50 mM Tris, pH 7.5, on an AKTA machine (Amersham Biosciences). The mutants were eluted with a 20 ml gradient of 0–2 M NaCl in 50 mM Tris, pH 7.5, and their elution time were measured by absorbance at 214 nm. Each experiment was repeated at least twice.

**Receptor Binding Assay—**Binding assays were performed in 96-well tissue culture plates in a total volume of 150 µl of binding buffer (0.5% BSA, 50 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM CaCl2). 300–19/mCXCR3 cells (4 × 10^9/well) were incubated with 0.04 nM 125I-labeled IP-10 (PerkinElmer Life Sciences, human IP-10), and increasing concentration of IP-10 mutants (5 × 10^-6 to 500 nM). After 90 min at room temperature with shaking the cells were transferred to 96-well filter plates (Millipore) previously soaked in 0.3% polyethyleneimine and washed four times with 200 µl of binding buffer supplemented with 0.5 µM NaCl. Radioactivity was counted after addition of scintillation fluid in a Microbeta counter (Wallac). The data were analyzed with GraFit (34). Each experiment was performed in duplicate and repeated at least twice. The mutants were eluted with a 20 ml gradient of 0–2 M NaCl. Radioactivity was counted after addition of scintillation fluid in a Microbeta counter (Wallac). The data were analyzed with GraFit (34). Each experiment was performed in duplicate and repeated at least twice.

**Calcium Mobilization—**Calcium mobilization was measured with a continuous 510 nm fluorescence measurement. The fluorescence was calculated by dividing the peak fluorescence at 340/380 nm and are presented as the relative ratio of fluorescence at 340/380 nm.

**Internalization of CXCR3—**Internalization of mCXCR3 was measured as previously described (35). Briefly, 300–19/mCXCR3 were resuspended in complete RPMI, 10% FCS, and various concentrations of wt or GAG-deficient cells expressing mCXCR3 were resuspended in their growth media with 1% FCS at 1 × 10^7 cells/ml and loaded with 5.0 μM acetoxyethyl ester of fura-2 (Molecular Probes, Eugene, OR) for 30 min at 37 °C in the dark. Cells were washed and resuspended at 5 × 10^6 cells/ml in calcium flux buffer (145 mM NaCl, 4 mM KCl, 1 mM NaHPO4, 0.8 mM MgCl2, 1.8 mM CaCl2, 25 mM HEPES and 22 mM glucose). Fluorescence readings were measured in a continuously stirring cuvette at 37 °C in a DeltaRAM (Random Access Monochromator) fluorimeter (Photon Technology International, Monmouth Junction, NJ). The data were recorded as excitation fluorescence intensity emitted at 510 nm in response to sequential excitation at 340 and 380 nm and are presented as the relative ratio of fluorescence at 340/380 nm. Fold induction (FI) of each stimulation was calculated by dividing the peak fluorescence ratio after stimulation by the peak fluorescence ratio during the 10 s before stimulation. Each experiment was repeated at least twice.

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comparison to known receptor and GAG binding sites of other CXC chemokines (Fig. 1). The first series of mutants included single point mutations to alanine or double mutations of adjacent basic residues to alanine. We chose a total of 16 mutants, including residues at the N terminus, identified for a number of chemokines to be important for signaling (36–38), residues along the 20s and 40s loop regions, shown for some chemokines to be important for receptor as well as GAG binding (39–41), and a set of basic residues at the C terminus, where the GAG binding site of IL-8 has been located (42). Based on the results of the first series of mutants, another six mutants were created. Residues of IP-10 mutated in this study are indicated with an arrow in Fig. 1.

Of the 22 mutants, one single point mutation, R38A, could not be expressed in a number of E. coli expression strains and was not further pursued. All the other mutants were successfully expressed in E. coli BL21 DE3 (pLysS) and purified from inclusion bodies by gel filtration, cationic exchange, and hydrophobic interaction chromatography to at least 95% homogeneity as determined by SDS-PAGE gels. N-terminal sequencing of the purified protein from the plasmid containing the unmutated wt IP-10 gene revealed that the N-terminal methionine was retained. We found this IP-10 to be fully active as compared with commercially available murine IP-10 from Peprotech, which has the initiating methionine cleaved off, in calcium flux, chemotaxis, and competitive receptor binding assays using 300–19 cells transfected with mCXCR3 (300–19/mCXCR3) as well as for chemotaxis and CXCR3 internalization of in vitro polarized murine Th1 cells (data not shown). The purified product from E. coli expressing the wt DNA was therefore called wt IP-10, and all other mutants for this study were similarly purified with the N-terminal methionine most likely retained. To eliminate the possibility that some of the effects seen with the IP-10 proteins could be due to LPS contamination, wt IP-10 as well as selected mutants were tested for the presence of endotoxin using a Limulus Amebocyte Lysate assay at a concentration of 1000 ng/ml and were found to be below the detection limit of 0.03 EU/ml. Mutants with reduced CXCR3 binding and reduced in vitro activity were analyzed for correct folding by near UV CD spectra. The spectra of all tested mutants were superimposable with the spectrum of wt IP-10, apart from mutant E40A, which displayed a clear shift. Mutant E40A was therefore excluded from the results.

Heparin Binding of IP-10 Mutants—The ability of the IP-10 mutants to bind heparin was analyzed by heparin affinity chromatography. Wt IP-10 eluted at a concentration of 0.96 M NaCl (Fig. 2). Of the first set of 14 mutants successfully expressed, 10 eluted at salt concentrations similar to the wt protein (difference of less than 0.10 M NaCl), including single and double mutations along the C-terminal helix. Mutations along the 20s loop region (also called N-loop) resulted in a larger difference; in particular, mutating Arg-22 had the largest impact of a single mutation. Mutations of the basic residues of the 40s loop also resulted in a reduction in heparin binding.

The specificity of heparin binding compared with electrostatic interaction was investigated by eluting the mutants from a cationic exchange column (SP HiTrap). The difference in salt concentration needed to elute mutants from the SP column in relation to the wt protein compared with the difference in elution from the heparin column confirmed that the residue Arg-22 had the highest specific binding to heparin. In contrast, residues along the C-terminal helix all displayed a negative differential (i.e. had at least as much a reduction in binding to the SP column as for the heparin column), indicating that the reduction in heparin binding due to mutation of these residues is mainly an electrostatic effect.

Based on the previous results, we created a second set of mutants, with a change charge of residues Arg-22 to glutamic acid and combining mutation of R22A with R20A, K47A, and R75E. In addition, the four basic residues of the C-terminal helix were mutated together, in a fashion similar to a mutant of PF4 that showed nearly no heparin binding affinity (43), to glutamic acid and glutamine yielding the mutant K71E/R72Q-K74Q/R75E (named “C-t mut”). This C-terminal mutant was also combined with the R22A mutation (C-tR22A).

Mutation of Arg-22 to a negatively charged glutamic acid further reduced the heparin binding affinity, as did combination with R20A and K47A (Fig. 2). These mutants displayed the highest amount of specificity for heparin binding, revealed by the differential in SP and heparin column elution. Although the double mutant of K74A/R75A had only a moderate effect on heparin binding, the combination of R22A with R75E still reduced the heparin binding. Similarly, K71E/R72Q-K74Q/R75E also showed a reduction of heparin binding of 0.29 M NaCl, and combining this mutation with R22A led to the largest decrease in heparin binding observed in this study. However, a comparison with binding to the SP column showed that this effect was mainly electrostatic in nature.

CXCR3 Receptor Binding—In a competitive binding assay using 300–19/mCXCR3 cells, wt IP-10 competed for the binding of 40 pg 125I-labeled human IP-10 with an IC50 value of 0.11 nM (Fig. 3), in agreement with published Kd values (44). Eight of the mutants (I12A, E28A, S33A, T44A, K62A, K66A, K71A/R72A, K74A/R75A) had IC50 values comparable to the wt protein. The N-terminal mutant R5A showed a 7-fold higher value, while mutant R8A had a 60-fold higher value. Mutant R22A, I24A, and K26A, which had shown lower heparin binding affinity, displayed 6-, 4-, and 2-fold lower receptor binding affinity, respectively. K46A/K47A, which had similarly reduced heparin binding, had a much more reduced CXCR3 binding affinity. The second set of mutants (R22E, R20A/R22A, R22A/K47A, R22A/R75E), all with further reduced heparin binding, had 15- to 60-fold reduced binding affinity for the receptor. Mutation of the four basic residues of the C terminus decreased the receptor binding 80-fold, and combination with the R22A mutation further reduced the binding another 10-fold, resulting in a nearly 1000-fold reduction in binding affinity.

Calcium Flux of IP-10 Mutants—All mutants were tested at concentrations of 200 and 1000 ng/ml for their ability to induce calcium mobilization in 300–19/mCXCR3 cells with a subsequent challenge of 200 ng/ml wt IP-10 to test the ability of the mutant proteins to desensitize the cells (Fig. 4). The eight mutants with CXCR3 binding and heparin binding comparable to wt IP-10 (I12A, E28A, S33A, T44A, K62A, K66A, K71A/R72A, K74A/R75A) all behaved equivalently to wt IP-10 in terms of calcium flux, chemotaxis, and internalization (data not shown) and will therefore not be listed in the remaining figures. The ability to cause calcium flux in 300–19/mCXCR3 cells followed a similar pattern for receptor binding. Mutant R8A did not induce calcium mobilization at concentrations of 200 or 1000 ng/ml (Fig. 4) or at 5000 ng/ml (data not shown) and did not desensitize the cells to a subsequent challenge of wt IP-10. Mutants R5A and R22A, with 5- to 6-fold reduced receptor binding affinity, triggered only a small calcium mobilization at 200 ng/ml but were more effective at 1000 ng/ml. Mutants with more reduced CXCR3 binding (R22E, R22A/K47A, R22A/R75E, C-t mut), all displayed strongly reduced ability to cause calcium mobilization even at 1000 ng/ml.

Chemotaxis Induced by IP-10 Mutants—The ability to cause chemotaxis of 300–19/mCXCR3 cells was related to the receptor binding and calcium mobilization data (Fig. 5) with some notable exceptions. Small reductions in receptor binding affin-
ity did not affect the chemotactic activity of the mutants as much as it did for calcium mobilization. Mutants R5A, R22A, and K26A, with only slightly reduced CXCR3 binding, displayed nearly wt chemotactic activity with similar potency and efficacy. Mutants R22E, R22A/K47A, R22A/R75E and C-t mut, which had reduced CXCR3 and heparin binding, exhibited both

Fig. 2. Heparin-Sepharose and cationic exchange chromatography of IP-10 mutants. 20 μg of IP-10 mutants were loaded onto either a 1 ml heparin HiTrap column (A) or a 1 ml SP HiTrap FF (cationic exchange) column (B) and eluted with increasing concentration of NaCl. C, summary of molarity of NaCl required to elute mutants from a heparin or SP column expressed as mean ± S.D. of two to four experiments. Results of the first set of mutants are presented in the upper part of C, results of second set in lower part of C. C-t mut, K71E/R72Q-K74Q/R75E, C-tR22A; C-t mut combined with mutation R22A.
reduced potency and efficacy for chemotaxis. Mutant C-tR22A was 6- to 7-fold less potent than wt IP-10 for chemotaxis, even at concentration of 5000 ng/ml. Mutant R8A did not cause chemotaxis up to concentrations of 5000 ng/ml.

Internalization of mCXCR3 Caused by IP-10 Mutants—All of the IP-10 mutants were analyzed for their ability to cause CXCR3 internalization. For this, 300–19/mCXCR3 transfected cells were incubated for 30 min at 37°C with various concentrations of IP-10, after which time the cells were stained on ice with an anti-mCXCR3 antibody and CXCR3 expression was analyzed by FACS. No internalization was observed with incubation at 4°C (data not shown). Wt IP-10 caused 68% internalization at 10 ng/ml and 92% at 1000 ng/ml (Table I). As seen for receptor binding and chemotaxis, mutant R22A was slightly less efficient than wt IP-10 in triggering internalization, while the mutants with further reduced CXCR3 binding displayed further reduced ability to cause internalization. Mutant R8A was the least effective mutant, triggering less than 30% internalization at 1000 ng/ml, while mutant C-tR22A, with 50-fold lower receptor binding affinity than R8A, caused nearly 50% internalization at 1000 ng/ml.

GAG-deficient CHO Cells—IP-10 mutants with significantly reduced heparin binding all displayed reduced receptor binding and reduced agonist activity. This could be due to the fact that heparin/GAG binding is promoting receptor binding and activation, as has been shown for other chemokines (25). Alternatively, the binding sites of CXCR3 and GAGs could be overlapping, thereby causing the mutants with reduced GAG binding affinity to also have reduced CXCR3 binding. To address this question, wt CHO cells as well as CHO cells deficient of GAGs (CHO 745) (33), were stably transfected with mCXCR3. Clones of wt and GAG-deficient CHO cells with similar CXCR3 expression levels were selected based on FACS analysis. Both wt and GAG-deficient CHO cells transfected with mCXCR3 responded with a similar rise in intracellular calcium concentration when stimulated with 200 ng/ml wt IP-10. The FI of the second dose is listed in parentheses, with a value of 1.0 indicating complete desensitization of the cells.

FIG. 3. Competitive CXCR3 binding assay. A, the binding of 125I-labeled IP-10 to 300–19/hCXCR3 cells was competed by IP-10 mutants (wt, R8A, R22A, R22E, C-tR22A). B, IC50 values were calculated for all IP-10 mutants and are expressed as mean ± S.E. from two to four experiments.

FIG. 4. Calcium flux of 300–19/mCXCR3 cells in response to IP-10 mutants. 300–19/mCXCR3 cells were loaded with 5.0 μM acetoxymethyl ester of fura-2, and fluorescence intensity emitted at 510 nm in response to sequential excitation at 340 nm and 380 nm was recorded. An overlay is shown of the relative ratio of fluorescence at 340/380 nm for selected mutants at 200 ng/ml (A) and 1000 ng/ml (B) initial stimulation with a subsequent stimulation of 200 ng/ml wt IP-10. C, the FI of calcium mobilization following each stimulation was calculated as described under “Materials and Methods,” and the mean values from at least two independent experiments are listed. The amount of desensitization of the cells after the primary dose of wt or mutant IP-10 is measured by a second stimulation with 200 ng/ml wt IP-10. The FI of the second dose is listed in parentheses, with a value of 1.0 indicating complete desensitization of the cells.
CHO cells. Even at lower concentrations of 50 ng/ml IP-10 there was no difference in calcium flux between the two cell types (data not shown). In a competitive binding assay, the binding of radiolabeled human IP-10 to wt murine IP-10 competing for the binding of the radiolabeled hIP-10 was 0.26 nM for wt CHO cells and 0.18 nM for GAG-deficient CHO cells, indicating that GAGs were essential for receptor binding. Given the possibility that GAGs could have a contribution in receptor binding and activation that could be counterbalanced by other factors in the GAG-deficient cells, we tested the ability of selected heparin binding reduced IP-10 mutants to compete for binding to CXCR3 in the GAG-deficient CHO cells. As shown in Fig. 6B, the IC<sub>50</sub> values for mutant R22E were similar for binding to the wt and GAG-deficient CHO cells expressing CXCR3, while IC<sub>50</sub> values for three experiments of 3.4 ± 1.4 nM and 3.3 ± 0.86 nM, respectively. The IC<sub>50</sub> values for mutants K46A/K47A and R20A/R22A were also similar for binding to wt and GAG-deficient CHO cells expressing CXCR3 (data not shown), suggesting that the mutants of the 20s and 40s loop regions have reduced CXCR3 binding independent from their reduced GAG binding affinity.

**DISCUSSION**

In the present study we used a mutagenesis approach to define the residues of IP-10 that mediate its interaction with CXCR3 and heparin. Residues Arg-20, Arg-22, Ile-24, and Lys-26 as well as Lys-46 and Lys-47 were found to constitute the main GAG binding domain, with mutation of Arg-22 resulting in the largest reduction of heparin binding affinity. In an homology model of mIP-10 based on the NMR structure of hIP-10 (45) created by the program Swiss-Model (46, 47), these residues form part of the loop region leading to and including the first residues of the first β-sheet (residues 20–26) and the spatially adjacent loop region after the second β-sheet (Lys-46, Lys-47), and are all along the same side of the protein (Fig. 7). Residues Arg-20, Arg-22, Lys-46, and Lys-47 line a groove that could potentially constitute the heparin binding site (Fig. 7C). The basic residues on the C-terminal helix when mutated in single or double point mutations had only a very modest effect on heparin binding. However, when the four basic residues Lys-71, Arg-72, Lys-74, and Arg-75 were mutated together with two changes to neutral and two changes to acidic amino acids, this resulted in a mutant with a greater reduction in heparin binding. Combining this quadruple mutation with a mutation at Arg-22 led to the largest decrease in heparin binding observed in this study. However, the reduction in heparin binding due to mutations along the C-terminal helix seems to be due to an electrostatic effect, whereas mutations along the 20s and 40s loop resulted in a heparin-specific binding reduction. It is therefore difficult to evaluate the contribution of the residues along the C-terminal helix in GAG binding. The findings are very similar to what was shown for PF4 (43), whereby NMR the main GAG binding site was found to be along residues Arg-20, Arg-22, His-23, Thr-25, Lys-46, and Arg-49. Double and even quadruple mutations of basic residues along the C-terminal α-helix of PF4 to alanine did not affect the heparin binding very much, whereas changes of four basic residues to two glutamines and two glutamic acids reduced the heparin binding 500-fold. Since the NMR structure of IP-10 only defines the
structure up to residue 70, we could not model residues 71–75, including the four basic residues, and cannot with confidence predict their position in relation to the other heparin binding residues. However, with an estimated 3.5–4 residues per helix turn, it is likely that residue 71 and 75 would be in close proximity to the 20s and 40s loop. Their mutation to negatively charged residues might therefore disturb the GAG binding site of IP-10, leading to the reduction in heparin binding observed for the K71E/R72Q-K74Q/R75E mutation, without those residues themselves being directly involved in heparin binding.

CXCR3 binding of the mutants was investigated with a competitive binding assay. As seen for other chemokines, residues at the N terminus (as seen for Arg-5 and Arg-8) just preceding the first cysteine are involved, though not critical, for CXCR3 binding. The 20s loop region (Arg-20, Arg-22, Ile-24) is also important for receptor binding, as are residues in the spatially adjacent 40s loop (Lys-46, Lys-47) (Fig. 7). Single or double mutations of residues along the C terminus did not have a significant impact on CXCR3 binding. However, the quadruple mutation Lys-72, Arg-74, Lys-74, Arg-75 with two charge changes did lead to a 100-fold reduced binding and, combined with mutation R22A, to a 1000-fold reduced binding. However, since the double mutations of the residues along the C terminus did not affect CXCR3 binding, it is unlikely that these residues are themselves involved in binding to CXCR3, but a double charge change might interrupt the nearby CXCR3 binding site. It is interesting to note that two of the main residues identified to be involved in CXCR3 binding, namely Arg-8 and Lys-46, are fully conserved in all murine and human CXCR3 ligands (see Fig. 1).

A recent study published the NMR structure of an obligate monomer mutant of human IP-10 (45). To investigate the interaction with CXCR3, a peptide consisting of the N-terminal residues 22–42 of CXCR3 was titrated into the IP-10 solution. Upon titration, changes in chemical shifts were observed for residues Val-7, Arg-8, Gln-17, Val-19, Gln-34, Arg-38, and Thr-44. Although based on a completely different technique, our study identified residues in similar regions on IP-10 interacting with CXCR3, namely residues in the N terminus and 20s loop. In addition, the NMR study identifies residue Arg-38 as involved in CXCR3 binding. In our study, mutation of Arg-38 was performed; however, the mutant did not express in bacteria. In addition, the NMR study also identified the 30s loop to be involved in the CXCR3 binding, based on the change in chemical shift seen for Gln-34. This residue is not conserved in murine IP-10, but in our study, mutation of Ser-33 did not affect CXCR3 binding. However, this could be due to the relatively small mutation of a serine to an alanine not interrupting the receptor ligand interaction. Lastly, residue Thr-44 showed a change in chemical shift in the NMR study, but as the authors state, this residue is actually buried and is not located on the same face of the protein as the other residues (45). Thr-44 was chosen for mutation in this study as it is considered conserved in all murine and human CXCR3 ligands, but its mutation to alanine did not impact CXCR3 binding or signaling. Therefore, compared with the NMR study, our mutational study identifies some similar regions (N terminus and 20s loop), as well as some different regions (40s loop, but not 30s loop) on IP-10 to be involved in CXCR3 binding.

The residues preceding the first cysteine have been shown for a number of chemokines to be critical for signaling. In particular, in the case of IL-8, the ELR motif is essential for signal transduction and mutations in this region completely abrogated function while only reducing receptor binding (37). Similarly, for MCP-1, N-terminal residues were found to be essential for signaling while being less important for CCR2 binding (38). In our study, we found residue Arg-8 of IP-10 to be critical for signaling through CXCR3. Mutation of this residue did not affect heparin binding and led to a 60-fold reduction of CXCR3 binding. Although other mutants with similarly reduced CXCR3 binding were still able to signal, mutation of Arg-8 could not induce any significant chemotaxis or calcium mobilization even at concentrations of 5000 ng/ml. Mutant R8A did induce a small amount of CXCR3 internalization, which has a distinct signaling pathway and is independent of Gαi protein signaling (35). The calcium flux data revealed that mutant R8A is not an antagonist as it did not block wt IP-10 calcium flux.

As schematized in Fig. 7, mutations along the 20s and 40s loop resulted in reduced heparin as well as CXCR3 binding. To address the question of whether the reduced ability to bind GAGs affected the CXCR3 binding of these mutants, wt CHO cells and a mutant CHO cells line deficient in xylosyltransferase and therefore lacking GAGs, were transfected with mCXCR3 and used to analyzed the IP-10 mutants. Wt IP-10 induced an equivalent amount of calcium mobilization in wt and GAG-deficient CHO cells expressing mCXCR3 and had similar IC50 values for both, showing that the GAGs on these transfected cells were not essential for CXCR3 binding and signaling. However, these cells are overexpressing CXCR3, and it could be that in biological settings with a lower CXCR3 surface expression, GAGs help to present IP-10 to the receptor.
Mutants R22E, R20A/R22A and K46A/K47A, which had reduced heparin and CXCR3 binding and signaling in 300–19 mCXC3 cells, showed the same 30- to 60-fold reduced CXCR3 binding for both wt and GAG-deficient CHO cells. This suggests that the 20s and 40s loop regions are indeed involved in CXCR3 binding, and therefore the CXCR3 and heparin binding sites are overlapping.

Although these experiments suggest that GAGs on cells overexpressing CXCR3 are not required for CXCR3 binding and signaling, GAGs on endothelial cells or other non CXCR3-bearing cells could still be important for retaining and sequestering IP-10 close to the site of secretion. In addition, GAGs might also be involved in biological functions of IP-10 on non-CXCR3-expressing cells. We hope to explore this hypothesis with mutants of IP-10 that have normal heparin binding affinity but reduced CXCR3 binding affinity.

In conclusion, our study has defined the N-terminal residue Arg-8 of IP-10 as being critical for signaling through CXCR3 and identified residues on the 20s and 40s loop as being important for CXCR3 binding. We also demonstrated that the GAG binding site of IP-10 is partially overlapping with the CXCR3 binding site. These mutants provide useful tools to further dissect the role of CXCR3 and GAG binding in the known biological activities of IP-10, including recruitment and activation of T cells and NK cells and inhibition of angiogenesis, fibrosis, and tumor growth. In addition, given the validation of the important role of IP-10 and CXCR3 in certain in vivo models of disease, such as allograft rejection (14, 17, 48), our study provides a structural basis for the rational design of models of disease, such as allograft rejection (14, 17, 48), and tumor growth. In addition, given the validation of the biological activities of IP-10, including recruitment and activation of T cells and NK cells and inhibition of angiogenesis, fibrosis, and tumor growth. In conclusion, our study has defined the N-terminal residue Arg-8 of IP-10 as being critical for signaling through CXCR3 and identified residues on the 20s and 40s loop as being important for CXCR3 binding. We also demonstrated that the GAG binding site of IP-10 is partially overlapping with the CXCR3 binding site. These mutants provide useful tools to further dissect the role of CXCR3 and GAG binding in the known biological activities of IP-10, including recruitment and activation of T cells and NK cells and inhibition of angiogenesis, fibrosis, and tumor growth. In addition, given the validation of the importance of IP-10 and CXCR3 in certain in vivo models of disease, such as allograft rejection (14, 17, 48), our study provides a structural basis for the rational design of models of disease, such as allograft rejection (14, 17, 48), and tumor growth. In conclusion, our study has defined the N-terminal residue Arg-8 of IP-10 as being critical for signaling through CXCR3 and identified residues on the 20s and 40s loop as being important for CXCR3 binding. We also demonstrated that the GAG binding site of IP-10 is partially overlapping with the CXCR3 binding site. These mutants provide useful tools to further dissect the role of CXCR3 and GAG binding in the known biological activities of IP-10, including recruitment and activation of T cells and NK cells and inhibition of angiogenesis, fibrosis, and tumor growth. In addition, given the validation of the importance of IP-10 and CXCR3 in certain in vivo models of disease, such as allograft rejection (14, 17, 48), our study provides a structural basis for the rational design of models of disease, such as allograft rejection (14, 17, 48), and tumor growth.

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