The Novel CXCL12γ Isoform Encodes an Unstructured Cationic Domain Which Regulates Bioactivity and Interaction with Both Glycosaminoglycans and CXCR4

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Background. CXCL12α, a chemokine that importantly promotes the oriented cell migration and tissue homing of many cell types, regulates key homeostatic functions and pathological processes through interactions with its cognate receptor (CXCR4) and heparan sulfate (HS). The alternative splicing of the cxcl12 gene generates a recently identified isoform, CXCL12γ, which structure/function relationships remain unexplored. The high occurrence of basic residues that characterize this isoform suggests however that it could feature specific regulation by HS. Methodology/Principal Findings. Using surface plasmon resonance and NMR spectroscopy, as well as chemically and recombinantly produced chemokines, we show here that CXCL12γ first 68 amino acids adopt a structure closely related to the well described α isoform, followed by an unfolded C-terminal extension of 30 amino acids. Remarkably, 60 % of these residues are either lysine or arginine, and most of them are clustered in typical HS binding sites. This provides the chemokine with the highest affinity for HP ever observed (Kd = 0.9 nM), and ensures a strong retention of the chemokine at the cell surface. This was due to the unique combination of two cooperative binding sites, one strictly required, found in the structured domain of the protein, the other one being the C-terminus which essentially functions by enhancing the half life of the complexes. Importantly, this peculiar C-terminus also regulates the balance between HS and CXCR4 binding, and consequently the biological activity of the chemokine. Conclusions/Significance. Together these data describe an unusual binding process that gives rise to an unprecedented high affinity between a chemokine and HS. This shows that the γ isoform of CXCL12, which features unique structural and functional properties, is optimized to ensure its strong retention at the cell surface. Thus, depending on the chemokine isoform to which it binds, HS could differentially orchestrate the CXCL12 mediated directional cell kinesis.

INTRODUCTION

Int.)CXCL12, also known as SDF-1 (Stromal cell-Derived Factor-1), belongs to the growing family of chemokines, a group comprising some fifty low molecular weight proteins, best known to mediate leukocyte trafficking and activation [1]. CXCL12, initially identified from bone marrow stromal cells and characterized as a pre-B-cell stimulatory factor [2], is constitutively expressed in cell migration and tissue homing of many cell types, regulates key homeostatic functions and pathological processes through interactions with its cognate receptor (CXCR4) and heparan sulfate (HS), to which most chemokines bind [17]. The authors have declared that no competing interests exist.

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primarily through ionic interactions. Anchored to various core proteins to form proteoglycans, these complex polysaccharides are ubiquitously found on the cell surface and within the extracellular matrix [18]. These molecules have a unique molecular design in which sulfated disaccharide units are clustered in specific domains of variable length and sulfation profile, providing the chain a large array of different protein binding sites [19]. HS are importantly implicated in the regulation of the proteins they bind, and have recently emerged as critical regulators of many events involving cell response to external stimuli. Current models suggested that HS enhance chemokine immobilization and forms haptotactic gradients of the protein along cell surfaces, hence providing directional cues for migrating cells [20], protects chemokines from enzymatic degradation [21], and promotes local high concentrations at the cell surface, facilitating receptor binding and downstream signaling [for review see [22]]. In vivo data support the view that, within tissues, CXCL12 is sequestered by HS [23].

CXCL12α binding to HS critically involves amino acids K24 and K27, which together with R41 form the essential part of the HS-binding site [24] and are distinct from those required for binding to CXCR4. Given that the minor δ, ε and φ isoforms lack any recognizable HS-binding motif in their carboxy-termini, it can be hypothesized that like CXCL12α, the K24-K27-R41 epitope recapitulates their ability to interact with HS. The situation could be radically different for the novel CXCL12γ isoform. It is indeed characterized by a distinctive 30 amino acids long C-terminal peptide, remarkably conserved between rodents and human, which contains as much as 18 basic residues (B, 9 of which being clustered into three putative BBXB HS-binding domains (Fig. 1A). The existence of carboxy-encoded HS-binding motifs suggests that this isoform could interact with enhanced affinity and/or different selectiveness with GAGs to accomplish specific functions. However, the structure/function relationships of this very peculiar CXCL12 isoform have not been explored yet. Here, we show that CXCL12α 1 to 68 domain adopts a structure closely related to the α isoform and has an unstructured C-terminal region. This domain reduces CXCR4 occupancy, but in contrast broadens the spectrum of GAG to which the chemokine binds. Moreover, it stabilizes the CXCL12α/HS complex and, in cooperation with the K24-R41 epitope, provides the chemokine with the highest affinity for GAGs ever observed for any chemokine.

RESULTS AND DISCUSSION

Wild type and mutants CXCL12 production

The CXCL12γ cDNA, obtained from Balb/C mouse brain mRNA was cloned and over expressed in E. coli, purified to homogeneity, and characterized by mass spectrometry, NMR and amino acid analysis. The preparation routinely yielded 4–5 mg of purified protein per liter of bacterial culture. Wild type and mutants CXCL12γ, β and γ, (Fig. 1A) were also produced by chemical synthesis and characterized by ion spray mass spectrometry and HPLC analysis. Final purity of all samples was found to be, on average, in the range of 90–95%. The biological activity (chemotaxis) of the recombinant chemokine and its chemically synthesized homologue was identical (data not shown).

CXCL12γ has a typical chemokine fold in the 1–68 domain and an unstructured C-terminal extension

CXCL12γ structure has been solved both by X-ray crystallography [25,26] and NMR spectroscopy [27]. The α and β isoforms structures are similar [28] but no information has yet been reported for CXCL12γ. To perform structural and binding, recombinant CXCL12γ was purified from cells grown in 15NH4Cl and 13C-glucose supplemented medium. Backbone resonances were assigned and the secondary structure content evaluated from 15N, 13C and 2H frequencies (TALOS [29]). The fold similarity of CXCL12γ and α was assessed by recording orientational informations N-H in Residual Dipolar Couplings (RDC) of partially aligned molecules in dilute liquid crystal [30], and NMR relaxation experiments were used to evaluate regions of flexibility.

The first 68 residues of CXCL12γ have a spectrum very similar to that of CXCL12α [29,31], enabling the identification of most residues by visual inspection. This was confirmed by the complete assignment of CXCL12α; residues, but K1, E73 and K84 (Fig. 1B). However, the assignment of CXCL12γ 69–98 remains tentative for the repeated KK motifs which present very similar backbone chemical shifts. Secondary structure prediction from the backbone chemical shifts indicated almost identical secondary structure content for CXCL12α and γ. Forty two N-H 15N RDCs, in the 10–64 domain of CXCL12γ were analyzed against CXCL12α, 33 of them showed an overall good correlation (Fig. 1C), which suggests that CXCL12γ 1–68 domain and CXCL12α adopt identical tertiary structure. CXCL12γ 1–68 relaxation parameters (R1, R2 and 15NH NOes) were highly similar to those observed for monomeric CXCL12α [31] with the residues 10–64 being well structured. Residues 69–98 behaved differently: they were clustered between 8 and 3.5 ppm in the 1H dimension, suggesting they were poorly ordered in solution (Fig. 1D). According to TALOS, only a few residues are predicted to adopt an extended conformation (Fig. 1A). Seven N-H 15N RDCs were observed in the γ extension between 2 and 7 Hz, presumably indicative of averaged RDCs due to important flexibility. This domain, with negative 15N−H NOes and low R1 and R2 relaxation rates compared to the protein core, experienced fast timescale dynamics, confirming it was highly disordered in solution. Together, these data show that the C-terminal peptide is disordered and has no major effect on the structure of the first 68 residues of CXCL12γ.

The prevalence of such non structured protein segments, recently became increasingly recognized [32]. These domains, known as intrinsically disordered or natively unfolded, usually feature a unique combination of low overall hydrophobicity and high net charge, a point that clearly characterize the CXCL12α C-terminal peptide. Proteins with such disordered regions are believed to performed critical functions, including molecular recognition through large and accessible interaction surfaces. In view of the highly basic nature of the CXCL12γ C-terminal domain, its disordered state, and the importance of GAG recognition for chemokine function, we then investigated the ability of CXCL12γ to interact with a variety of GAGs, including heparin (HP), HS, and dermatan sulfate (DS), and compared it to that of CXCL12α, β, which C-termini are distinct.

CXCL12α, β and γ differently bind to GAGs

To determine the GAG binding ability of CXCL12α, β and γ isoforms we adopted a solid phase assay, in which reducing end biotinylated HP, HS or DS were captured on top of a streptavidin coated sensorchip, a system that mimics, to some extent, the cell membrane-anchored proteoglycans. Surface plasmon resonance (SPR) real time monitoring was exploited to measure changes in refractive index caused by the binding of chemokines to each of the immobilized GAGs.

Binding curves, obtained when the CXCL12 isoforms were flowed over the HP, HS and DS surfaces, showed marked differences (Fig. 2). These experiments first indicated that while CXCL12γ interacts with HP, HS and DS, CXCL12γ and β only recognize HP and HS, suggesting that the C-terminal domain,
which characterizes the γ isoform, enables the chemokine to extend the range of GAGs to which it binds. Visual inspection of the sensorgrams also showed major differences during the dissociation phase. CXCL12γ dissociated from the immobilized GAGs rapidly (binding curves returned to the baseline within a minute), while CXCL12δ formed tight complexes, and CXCL12β displayed an intermediate behaviour. Preliminary analysis of the binding curves indicated that the binding rates were dominated by mass transfer, and global fitting of the binding curve returned values with low significances (see below). Because we generated data in which the association phase was allowed to proceed to equilibrium, affinity data were derived independently from the kinetic. By plotting R₀/C against R eq for different concentrations of chemokine (in which R eq are the steady state values at equilibrium and C the concentrations of injected chemokine), straight lines were obtained (data not shown) which slopes, corresponding to the equilibrium constant Kd, are reported in Table 1. These analyses demonstrated that CXCL12γ interacts with GAGs with an unprecedented propensity, featuring a 2 log increase compared to CXCL12α, and suggesting a strong participation of the C-terminal domain in the binding reaction.

**Heparin derived oligosaccharides interact with CXCL12γ C-terminal domain and reduce its mobility**

In view of the above data, which support the existence of additional GAG binding sites within the C-terminal domain of CXCL12γ, we performed titration experiments of 15N-CXCL12γ with different HP derived di-(dp2), tetra-(dp4) and octa-(dp8) saccharides. The CXCL12γ/oligosaccharide interactions were in fast exchange regime compared to NMR chemical shift timescale, typical of interactions in the µM-mM Kd range. Interaction with
dp4 reached saturation, with an apparent Kd of about 250 μM. Several resonances in the γ extension were highly perturbed upon interaction (Fig. 3A). However, they could not be individually followed during titrations and backbone resonance assignment was performed on the 15N-13C-CXCL12γ/dp4 complex.

Interactions of dp2, dp4 and dp8 with CXCL12γ revealed two binding domains on the protein (Fig. 3). On the CXCL12γ core region, the most perturbed residues form a continuous surface, from R20 to R41 (Fig. 3D), including V23, K24, A40, and N45. This binding surface suggested an oligosaccharide orientation more or less perpendicular to the β sheet which differs from the previously described orientation of a dp12 in complex with a CXCL12α dimer. In that case, the oligosaccharide also binds K24 and R41 but is aligned along the first β strand [24]. On the C-terminal extension, most of the residues were perturbed by the interaction in particular residues 83 to 97. Mab 6E9, which epitope consists of residues 78–80, still bound to the CXCL12γ/GAG complex (data not shown), further supporting the importance of the distal part of the C-terminus. Backbone chemical shifts from CXCL12γ/dp4 complex did not reveal any secondary structural changes compared to the free protein and no appearance of secondary structure elements in the C-terminal extension. 15N-1H heteronuclear NOEs on the complex (data not shown) indicated however a significant decrease in mobility upon dp4 binding for the γ extension with positive NOe values for residues 82 to 89. A maximum NOe value around 0.2 for Q87 (data not shown) suggested nevertheless that, even in complex with HP derived oligosaccharides, the γ extension still exhibits important flexibility.

The C-terminal domain and the binding sites in the core structure of CXCL12γ differently contribute to the binding

To further analyze the respective GAG binding contributions of the core region and the C-terminal domain of CXCL12, mutations were introduced in both parts of the chemokine (see Fig. 1A) and their binding profiles were analyzed using the SPR assay (Fig. 4). As mentioned above, simultaneous fitting of the association and dissociation phases was not possible, presumably due to fast on rate which causes strong mass transport limitation during the association phase (data not shown), and possibly rapid rebinding of the dissociated molecules during the dissociation phase. The dissociation rates (koff) were thus measured at the beginning of the dissociation phase (where rebinding is limited because the number

| Table 1. Equilibrium dissociation constant of CXCL12 for HP, HS and DS |
|-------------------|-------------------|-------------------|
|                   | HP                | HS                | DS                |
| CXCL12 α          | 93 ± 6.1          | 200 nM ± 14       | No binding        |
| CXCL12 β          | 24.7 nM ± 2.6     | 53 nM ± 2.7       | No binding        |
| CXCL12 γ          | 0.91 nM ± 0.07    | 1.5 nM ± 0.2      | 4.8 nM ± 0.04     |

The equilibrium levels of bound CXCL12 were extracted from the sensorgrams of Fig. 2 at the end of the association phases (apart from the lowest CXCL12 concentrations, which in some cases did not reach equilibrium) and used to calculate the dissociation constant (Kd), using the Scatchard plot. Results are expressed in nM as means ± SEM of 3 to 7 experiments.

Figure 2. Analysis of CXCL12 binding to HP, HS and DS. SPR sensorgrams measured when CXCL12 were injected over HP, HS or DS activated sensorchips. The response in RU was recorded as a function of time for CXCL12α (26 to 300 nM), β (13 to 150 nM) and γ (2.6 to 30 nM).
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of free immobilized GAGs remains low) and the on rates \( \left( k_{on} \right) \) were then calculated using the equilibrium dissociation constant \( \left( k_{off}/K_d \right) \). Results are indicated in Fig. 5 and show that the C-terminal domain, while having limited effect on the on rate, essentially determines the velocity at which the formed complex dissociates. This is particularly marked for the \( \gamma \) isoform, which dissociates from HP with a \( k_{off} \) of 0.0019 M\(^{-1}\)s\(^{-1}\) compared to 0.111 M\(^{-1}\)s\(^{-1}\) for CXCL12\(\beta\) and 0.0204 M\(^{-1}\)s\(^{-1}\) for CXCL12\(\beta\). In agreement with these observations, mutations of the 3 basic residues present at the C-terminus of the \( \beta \) isoform (\( \beta\)-m1) did not change the on rate, but increased the off rate to a value of 0.098 M\(^{-1}\)s\(^{-1}\), thus resulting in a behavior very close to that of CXCL12\(\beta\) (Fig. 5), with an overall affinity of 125 nM for HP and 192 for HS (compare with results in Table 1). Mutations in the core region (K24S/K27S), that completely abolished CXCL12\(\beta\) binding to HP/HS [24], were also introduced in \( \beta\)-m1 to yield a new mutant (\( \beta\)-m2; see Fig. 1). As expected, \( \beta\)-m2 did not bind anymore to GAGs.

Similarly, the effect on GAG binding of mutations introduced in the C-terminal domain of CXCL12\(\gamma\) was analyzed. Amongst the 18 basic residues of this domain, 9 were changed for Ser which removed the 3 typical HP binding clusters (Fig. 1A). Preliminary analysis performed with C-terminal synthetic peptides (residues 69–98) indicated that the wild type sequence required 0.88 M NaCl to be eluted from a HP affinity column, while the mutant peptide eluted at 0.28 M NaCl. This mutant peptide did not show any binding up to 200 nM using the SRP assay, demonstrating that these mutations very strongly decreased its binding capacity (data not shown). The GAG binding profile of the mutated full length chemokine (\( \gamma\)-m1, which includes these 9 mutations), was characterized. We observed that this mutant did not bind anymore to DS. This supports the view that the net charge of the CXCL12\(\gamma\) isoform C-terminal domain was involved in the broad GAG binding activity. As could have been anticipated, \( \gamma\)-m1 displayed an increased dissociation rate compared to the wild type chemokine (Fig. 5A), confirming the role of the C-terminal domain in the complex stability. The equilibrium dissociation constant for HP of this mutant was 10.4 nM (32 for HS). Thus, although this C-terminal domain by itself has a highly reduced binding capacity, the full length molecule still interacts quite strongly with HP and HS, suggesting a predominant role for the core domain. Consistently with this hypothesis, additional mutations in the core structure (\( \gamma\)-m2) dramatically decreased HP and HS binding, supporting further the critical importance of the core domain binding site for the interaction. CXCL12\(\alpha\)/HP complex displayed an half live \( \ln[0.5]/k_{off} \) of 6 seconds, while CXCL12\(\gamma\)/HP complex was characterized by a half life of 350 seconds (Fig. 5B). Together, these data show that few key amino acids of the structured domain of CXCL12\(\gamma\) (in particular K24/27) constitute a strictly required binding site while, a number of positively charged residues of the unfolded C-terminus appears to primarily functions in stabilizing the formed complex.

Such different contributions between the two domains could be explained by the fact that electrostatic interactions are not always energetically positive. Favorable coulombic interactions formed in a final complex can be some times largely offset by the desolvation cost associated with the binding process [33], an effect that could occur within the unfolded and largely solvent accessible C-terminus of CXCL12\(\gamma\). DNA-binding domains frequently have N- or C-terminal extensions, enriched in basic residues, and disordered in solution. The contribution of such basic tails, which increase the affinity for target DNA, has been studied in the context of protein-DNA interaction [34], but to our knowledge this has not yet been described for protein-GAG complex. In any case, the present findings support the view that for CXCL12\(\gamma\), a large and unstructured C-terminal domain functions as an accessory “binding cassette” which, in cooperation with a restricted and well defined binding site in the core structure provides very tight binding to GAGs.

**CXCL12\(\gamma\) displays enhanced binding to cell surface expressed HS compared to CXCL12\(\alpha\)**

To investigate whether HS, in the context of the cell surface, also interacted more efficiently with CXCL12\(\gamma\) than with CXCL12\(\alpha\), we then compared the adsorption of these two isoforms on CXCR4 negative CHO cells by flow cytometry. The monoclonal antibody K15G, which recognize an epitope outside the HS binding site and present in all CXCL12 isoforms [35] were used for this purpose. Data are reported in Fig. 6, and show that binding to wild type CHO-K1 cells was greatly enhanced for CXCL12\(\gamma\) compared to CXCL12\(\alpha\). In particular, at low concentration (50 nM), CXCL12\(\alpha\) did not display significant binding, while the \( \gamma \) isoform bound strongly to the cell surface, in agreement with the Biacore data (Fig. 2). These interactions were strongly reduced on HS deficient CHO-pgsD677 cells, demonstrating the importance of HS in the binding.

**CXCL12\(\gamma\) displays reduced binding to- and signaling through- CXCR4**

To analyze the binding of CXCL12\(\gamma\) to CXCR4, we set up an assay, in which we compared the ability of the \( \alpha \) and \( \gamma \) isoforms to compete with \( ^{125}\)I-labeled CXCL12\(\alpha\). This was performed on T lymphoblastoid cell lines (CEM or A3.01), which do not express expressed HS compared to CXCL12\(\alpha\) in particular, (Fig. 7A). In agreement with this
observation, CXCL12γ has a reduced ability to stimulate intracellular calcium mobilization compared to the α isoform (Fig. 7B).

The large amount of GAGs usually found at the cell surface, the reduced affinity of CXCL12γ for CXCR4 and its very high affinity for HS, suggest that within tissues the γ isoform might be predominantly in a bound form, associated to GAGs, and either stabilized to prevent proteolytic degradation and/or immobilized to allow continued and localized stimulation of cells.

Conclusion

The binding of proteins to GAGs is the prerequisite for a large number of cellular processes and regulatory events. The chemokine system, in particular, strongly depends on HS, which

Figure 4. Analysis of wild type and mutant CXCL12 binding to immobilized GAGs. Binding of wild type and mutant CXCL12 were recorded as in Fig. 2. CXCL12α (26 to 300 nM), β, β-m1, β-m2 (13 to 150 nM), γ, γ-m1, γ-m2 (2.6 to 30 nM) were injected over GAG activated sensorchips and the response in RU was recorded as a function of time.
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are believed to ensure the correct positioning of chemokines within tissues.

In this report, we have shown that CXCL12γ, a new splice variant of CXCL12, displays an unusually high affinity for GAGs and investigated the structural determinants involved. The first 68 amino acids of the chemokine, common to all CXCL12 isoforms, comprised both the CXCR4 binding domain and a first, well defined, HS specific binding site. To this common platform is added, by alternative splicing of the cxcl12 gene, different peptides which contain a second GAG binding domain, limited to 4 additional residues for CXCL12β but as long as 30 residues for CXCL12γ. This domain, which remains unfolded, appeared to mainly function by stabilizing the chemokine/HS complex. This, in combination with the structured first HS binding site, provides the protein with an unprecedented high affinity for HS. Interestingly, it has been described that polypeptide segments generated by alternative splicing are mostly intrinsically disordered [36]. This has been thought as a way to generate functional diversity without structural modification or complication. Our present findings fit well with this proposed mode of action. Thus, by encoding a singular domain, bearing the CXCR4 binding site, CXCL12 may display distinct regulatory functions. The observation that the different CXCL12 isoforms mostly differ by their ability to interact with GAGs, offers an unprecedented opportunity to ascertain the importance of chemokine/GAG bindings in the regulation of in vivo cell migration. Regarding CXCL12γ, the remarkable conservation within mammalians, of its entire C-terminal sequence is intriguing for a domain which presumably essentially triggers electrostatic interactions, and argues in favor of an important role played by this isoform. The observation that GAGs trigger a rapid and almost irreversible accumulation of CXCL12γ suggests that within tissues it should exist essentially in a bound form in nearby cells, presumably to allow continued and localized cellular stimulation. These data are compatible with a selective role of this isoform, and indicate that GAGs could be critical in orchestrating the CXCL12 mediated migration of cells, depending on the chemokine isoform and the nature of the GAGs to which it binds, either during development or post-natal life.

**MATERIALS AND METHODS**

**CXCL12 production and characterization**

Murin *CXCL12γ* cDNA was inserted in a pET17b (Novagen) expression vector between NdeI and SpeI restriction sites, and checked by DNA sequencing. CXCL12γ was overexpressed overnight in *E. coli* BL21 (DE3) cells, with 0.4 mM IPTG, either in LB or M9 minimal medium supplemented with 15NH4Cl and 13C or 13C-glucose for isotopic enrichment. After 30 minutes of sonication at 4°C in 50 mM Tris pH 8.0 (buffer A), inclusion bodies were pelleted (20000g for 15 minutes) and washed with buffer A supplemented with 2M urea and 5% Triton X100, then diluted 4 times with buffer A and finally with buffer A. Inclusion bodies were solubilised for 15 min at 50°C in buffer A with 7.5 M GdCl3 and 100 mM DTT. Refolding was performed by rapid dilution with buffer A up to 1 M GdCl3. The mixture was gently stirred overnight at 4°C after addition of Complete protease inhibitors (Roche), then diluted 4 times with buffer A and loaded onto a 3 ml Source S column (Amersham) equilibrated in 20 mM Na2HPO4 pH 6.0. CXCL12γ was eluted with a NaCl gradient, concentrated and further purified on a G75 gel filtration column (Amersham) in 20 mM NaH2PO4, 150 mM NaCl pH 6.0. Purified material was analyzed by MALDI mass spectrometry and quantified by amino acids analysis. Wild type and mutants CXCL12α, β and γ were also produced by chemical synthesis, using the Merrifield solid-phase method and fluorenylmethoxycarbonyl chemistry, as described [24].

**Figure 5.** Association and dissociation rate constant of the CXCL12-GAG interaction. (A) Graphical summary of the data generated from the sensorgrams of Fig. 4, in which association (kon) and dissociation (koff) rate constants of CXCL12α (open circle), β (grey circle), β-m1 (grey square), γ (black circle) and γ-m1 (black square) for HP were determined as described. Differences were essentially observed along the koff axis. (B) Dissociative half live of the different CXCL12/HP complexes.

**Figure 6.** Flow cytometry analysis of CXCL12 interaction with cell surface GAGs. CHO-K1 parental cells (squares) or HS-deficient CHO-pgsD677 cells (triangles) were incubated with the indicated concentrations of CXCL12α (open symbols) or γ (close symbols) and, after extensive washing to remove free chemokine, were labelled with K15C mAb and analyzed by flow cytometry.

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Preparation of heparin derived di- tetra- and octasaccharides
Porcine mucosal HP was depolymerized with heparinase I. The digestion mixture was resolved from di-(dp2) to octa-(dp10) decasaccharide, and dp2 to dp8 were further purified by strong-anion-exchange HPLC as described [37].

NMR experiments
NMR experiments were recorded at 30°C on Varian spectrometers (600 ENova, 600 DD or 800 MHz with cryoprobe), processed with NMRpipe and analyzed with NMRview. CXCL12γ backbone assignment and relaxation experiments were recorded on 1 mM 15N-13C sample in 20 mM NaH2PO4 pH 5.7, 10% D2O, 0.01% NaN3 with protease inhibitors at 600 MHz. HNCA, CBCA(CO)NH and HNCO, 15N-1H NOes and T2 recorded on 1 mM 15N-13C sample in 20 mM NaH2PO4 pH 5.7, CXCL12 processed with NMRpipe and analyzed with NMRview.

Binding of CXCL12 to CXCR4 and cell surface HS
CEM cells (10⁷ cells/ml) were incubated with 0.25 nM of 125I-CXCL12γ (Perkin-Elmer, 2200 Ci/mmol) and a range of concentrations of unlabelled CXCL12 (α, γ or γ-m1) in 100 μl of PBS for 1h at 4°C. Incubations were stopped by centrifugation at 4°C. Cell pellets were washed twice in ice-cold PBS, and the associated radioactivity was counted. For measuring the ability of CXCL12 to interact with cellular HS, the CXCR4 negative CHO-K1 or HS-deficient CHO-pgsD677 (ATCC) were incubated with the chemokine and after removal of unbound proteins, were labelled with an anti-CXCL12 mAb (clone K15C) and a PE-conjugated secondary antibody. Immunolabelled cells were analysed by flow cytometry using a FacsCalibur (BD Biosciences).

Intracellular calcium release responses
Intracellular calcium measured in CXCR4-expressing cells loaded with fluo-4-AM (Interchim) was conducted in a Mithras LB 940 counter (Berthold Technologies). Briefly, A3.01 cells were incubated for 45 min at 37°C in the load buffer (10 mM Hapes, 137.5 mM NaCl, 1.25 mM CaCl₂, 1.25 mM MgCl₂, 0.4 mM NaH₂PO₄, 1 mM KCl, 1 mM Glucose) with 0.1% of pluronic acid and 0.5 mM of Fluor-AM (10⁶ cells/mL). After a washing step, cells were suspended in load buffer at a final concentration of 2×10⁶ cells/mL and stored at 4°C. For intracellualr calcium measurements, aliquots of cells (2×10⁶ cells) were preincubated at 37°C for 1 min, then placed in a 96-well flat bottom plate. Fluorescence emission was recorded at 535 nM (excitation at 485 nM) every second before (basal fluorescence) and after programmed injection of different concentration of the ligands. Maximum and minimum fluorescence values were determined after addition of Triton X-100 and EDTA, respectively. Data are expressed as fluorescence increment rate after ligand addition.
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