Published in final edited form as:

Anal Biochem. 2010 December 1; 407(1): 134–140. doi:10.1016/j.ab.2010.08.005.

A competitive binding study of chemokine, sulfated receptor, and glycosaminoglycan interactions by nano-electrospray ionization mass spectrometry

Connie H. Jen and Julie A. Leary

Department of Molecular and Cellular Biology, University of California, Davis, California, 95616

Abstract

Chemokines are secreted proteins that play roles in inducing chemotaxis, extravasation, and activation of leukocytes associated with inflammatory or homeostatic processes. Tyrosine sulfation of the chemokine receptor has been shown to be important for binding and signaling. We have applied a mass spectrometry method to measure the contribution of this posttranslational modification to binding of its ligand chemokine. Using Nano-electrospray time-of-flight mass spectrometry, we determined the association constant of chemokine, CCL7 with CCR2, monosulfated CCR2, and disulfated CCR2 peptides to be $1.1 \times 10^4 \text{ M}^{-1}$, $3.9 \times 10^4 \text{ M}^{-1}$, and $4.0 \times 10^5 \text{ M}^{-1}$, respectively. To our knowledge, this is the first reported association constant measurement between a protein and sulfated peptide using mass spectrometry. Furthermore, nano-ESI MS was used to characterize noncovalent binding interactions between CCL7, Arixtra (a pentasaccharide GAG analog) and disulfated CCR2 peptide. A lack of observable ternary complex formation prompted investigation of competitive binding. Results in this study suggest that CCR2 competes partially with GAG for CCL7 binding and that disulfated CCR2 peptide has a higher binding affinity than Arixtra, which correlates with data from association constant measurements for CCL7-disulfated CCR2 and CCL7-Arixtra.

Keywords

C-C motif chemokine 7 (CCL7); C-C chemokine receptor type 2 (CCR2); Arixtra; glycosaminoglycan (GAG); tyrosine sulfation; nano-electrospray ionization (nano-ESI); mass spectrometry; association constant (K_a); noncovalent complex

Introduction

The chemokine family is composed of small (8–10 kDa) secreted proteins that have critical roles in leukocyte recruitment and extravasation [1]. Leukocyte extravasation occurs during routine immune surveillance, inflammation, or development. Key steps during extravasation are: weak attachment and rolling of leukocytes along the endothelium; activation of leukocytes by chemokine binding; firm adhesion of the activated leukocytes to the

© 2010 Published by Elsevier Inc.

*Corresponding Author: Julie A. Leary, Briggs Hall 130, One Shields Ave., Davis, CA 95616, Telephone: (530) 752-4685, Fax: (530) 752-3085, jaleary@ucdavis.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

endothelium; and transmigration of leukocytes through the blood vessel wall to the underlying tissue [2]. Chemokines induce activation of a leukocyte for migration by binding with and signaling through a specific G-protein-coupled receptor expressed on the surface of a leukocyte [3]. To produce directional signals for cell migration, concentration gradients of chemokines must be formed. The establishment of cell surface gradients is thought to involve binding to glycosaminoglycans (GAGs) on the endothelial-cell surface, specifically heparan sulfate [4]. Evidence for the necessity of chemokine binding to GAG for leukocyte recruitment was demonstrated by Proudfoot et al. Chemokines with mutations in amino acids that mediate GAG binding induced chemotaxis *in vitro*, but they failed to recruit leukocytes *in vivo* in intraperitoneal recruitment assays [5].

Recent studies have shown that tyrosine sulfation of the N-terminal domain of chemokine receptors plays an important role in chemokine recognition and interaction. For example, sulfated tyrosine residues in the N-terminal domain of chemokine receptors CCR5 [6;7;8], CXCR4 [9;10], CCR2B [11], CCR8 [12], and CXCR3 [13] have been shown to be required for optimal ligand binding. By the use of sulfate labeling and tyrosine mutations, however, the studies mentioned above do not directly distinguish between the functional role of the sulfate modification and that of the tyrosine residue itself. To directly investigate the functional role of sulfated tyrosines, NMR has been used for comparison of binding affinities between sulfated and unmodified forms of peptides derived from the N-terminal regions of CXCR4 [10;14], CCR5 [15] and CCR3 [16] to their respective chemokine ligands.

Traditionally the association constant of a protein-ligand noncovalent complex can be determined by several methods including NMR, surface plasmon resonance, fluorescence and isothermal titration calorimetry (ITC) [17]. NMR has the advantage of providing information about probable sulfate tyrosine binding sites on the chemokine, but drawbacks include requirement of isotopic labeling of the chemokines and large sample consumption (approximately 0.25 mg of chemokine and 0.2 mg of the peptide) [10;14;15;16]. Isothermal titration calorimetry is an established, direct and accurate solution-based method for determination of dissociation constants over a wide range of affinities (millimolar to nanomolar) for protein-ligand interactions [18]. However, ITC also requires large quantities of samples [18], which are not always available. An advantage of nano-electrospray (nano-ESI) MS is low sample consumption; typically a sample solution of 0.5 μ L and 5 fmol of analyte is sufficient for nano-ESI [19;20]. Other advantages include minimum requirements for sample preparation, ability to directly obtain stoichiometric information about the protein-ligand interaction, and rapid data analysis.

Mass spectrometry has become a valuable analytical tool for studying noncovalent biological complexes at near-physiological conditions. Association constants determined by ESI-MS for various noncovalent complexes have been reported to be in good agreement with values using solution based methods [21;22;23;24;25;26;27]. Nevertheless, there are instances in which gas phase affinities do not reflect those measured in solution particularly for complexes that are based on nonpolar interactions [28;29]. Recently, nano-ESI MS has been applied to investigate protein-protein and protein-ligand noncovalent complexes, and nano-ESI is thought to better reflect the in-solution equilibrium [30]. The smaller initial spray droplets formed during nano-ESI require less solvent evaporation, which may influence equilibrium prior to ionization [31;32;33].

The current study was designed to demonstrate that nano-ESI TOF MS can yield reliable K_a values for chemokine and sulfated chemokine receptor peptides. Mass spectrometry was used to monitor the equilibrium for the solution-phase titration of chemokine CCL7 and sulfated CCR2 N-terminal peptide. Given the assumption that ionization does not notably

affect the equilibrium, the intensity of the complex can be compared to the intensity of the free protein for each ligand concentration. To our knowledge, this is the first application of a nano-ESI MS approach to measure binding affinities for a protein and sulfated peptide noncovalent complex.

Further nano-ESI MS experiments examined the relationship between Arixtra, CCL7, and disulfated CCR2 peptide binding. Binding interactions between GAGs and chemokine receptors have been shown to be crucial for normal chemokine function [1;4]. It has been hypothesized that a possible mechanism of action of cell surface GAGs is to present the chemokine to its receptor, and this presentation requires the formation of a chemokine, GAG, and receptor ternary complex [34]. For this to occur the binding sites for the receptor and GAG need to be spatially distinct and uncompetitive in terms of binding [34]. It has been debated whether a short-lived ternary complex forms between the receptor, chemokine, and GAG or whether their binding is competitive. Arixtra and disulfated CCR2 peptide (Fig. 1) were used as the minimal representative portions of GAG and the receptor that bind to chemokines at measureable affinities. Nano-ESI MS can be used to provide new insights to understanding the nature of these noncovalent binding interactions.

Materials and Methods

Materials

Recombinant human CCL7 was expressed as previously described in Yu et al [35]. Disulfated CCR2 21-30 (TTFFDY(SO₃H)DY(SO₃H)GA), monosulfated CCR2 21-30, and CCR2 21-30 (TTFFDYDYGA) were purchased from Biomer Technology (Pleasanton, Ca). Arixtra (fondaparinux sodium) was obtained from GlaxoSmithKline (Research Triangle Park, NC). Heparinase I was purchased from Seikagaku (Tokyo, Japan).

Glycosaminoglycan Sample Preparation

Arixtra was desalted using 1000 MWCO dispo-biodialyzer (The Nest Group, Southborough) in preparation for mass spectrometry analysis. One hundred microliters of approximately 2.9 mM Arixtra were dialyzed against deionized water for three days.

An enzyme-coupled UV assay was used to determine the concentration of desalted Arixtra as previously described in Yu et al [36]. Briefly, Arixtra is cleaved by heparinase I to yield an unsaturated disaccharide and a trisaccharide [37]. The enzymatically generated double-bond on the disaccharide strongly absorbs at 232 nm. The concentration of disaccharide was then directly correlated to the original concentration of Arixtra.

Dissociation Constant determination by Nano-ESI oa-TOF MS

Experiments were performed on the LCT Premier™ orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer (Waters Corporation, Milford, MA) equipped with a nano-electrospray ionization (nano-ESI) source. Nano-ESI was performed using borosilicate glass capillaries (1 mm o.d., 0.78 mm i.d., Sutter Instrument Company, Novato, CA) that were pulled and coated with gold. The capillaries were pulled on a Sutter P-97 Flaming/Brown micropipette puller, and gold coated using a Quorum Technologies SC7640 sputter coater (Ringmer, East Sussex, UK). The mass spectrometer was operated in positive ion mode with a capillary voltage of 1.0 kV, a sampling cone voltage of 20 V, and a source temperature of 40°C.

Non-covalent complexes were prepared from stock solutions of CCL7 and ligand(s) in 125 mM ammonium acetate, pH 6.8. A titration experiment was utilized for the MS dissociation constant determination. The concentration of CCL7 was maintained at 10 μM and CCR2

peptides were added at the following concentrations: 7.5, 10, and 12.5 μM . For each tip, 2 μL of analyte solution was used. Multiple measurements were taken for each given concentration to obtain R values. These R values were determined from averaging measurements from five identically pulled nano-ES tips, and for each nano-ES tip three sequential measurements were acquired under identical conditions.

For competitive binding experiments, the concentrations of CCL7 and Arixtra were each kept constant at 10 μM , and di-sulfated CCR2 peptide was varied using the following concentrations: 0.5, 1, 2.5, 5, 7.5, and 10 μM .

Spectra were acquired for the duration of one minute and were smoothed (3×5 channels). Assuming that the ionization does not affect the equilibrium and that ionization efficiencies are similar for CCL7 and for ligand-bound CCL7, the integrated peak areas directly correlate with solution concentrations. Using equations from Wang et al. [38], chemokine-ligand binding equilibrium constants were calculated using the derived solution concentrations as shown in Eq. 1; I_{CCL7} corresponds to the mass spectral peak area for all charge states of free CCL7, and $I_{\text{CCL7-L}}$ corresponds to the sum of all charge state peak areas of the CCL7-ligand complex. The ratio (R) of ion intensities of the bound chemokine and free chemokine ions includes all observed charge states (n). For each ligand concentration used, the K_a was directly calculated based on Eq. 1, and the overall reported K_a was taken from the average measurements for the three different ligand concentrations.

$$\begin{aligned} \text{CCL7} + \text{L} &\rightleftharpoons \text{CCL7} \cdot \text{L} \\ K_a &= \frac{[\text{CCL7-L}]_{\text{equil}}}{[\text{CCL7}]_{\text{equil}} [\text{L}]_{\text{equil}}} \\ R &= \frac{[\text{CCL7-L}]_{\text{equil}}}{[\text{CCL7}]_{\text{equil}}} = \frac{\sum_n [I^{n+}_{(\text{CCL7-L})}]}{\sum_n [I^{n+}_{(\text{CCL7})}]} \\ K_a &= \frac{R}{[\text{L}]_t - \frac{R * [\text{CCL7}]_t}{1+R}} \end{aligned} \quad (\text{Eq. 1})$$

Results and Discussion

K_a measurements for CCL7 and Arixtra

Using Nano-ESI oa-TOF mass spectrometry, the association constant for the CCL7-Arixtra complex was determined by titration experiments. The association constant was measured by maintaining the concentration of CCL7 at 10 μM and titrating in Arixtra at 7.5 μM , 10 μM , or 12.5 μM . The resulting mixture was analyzed directly by nano-ESI TOF MS. A 1:1 complex was observed between CCL7 and Arixtra (Figure 2), which is consistent with the reported stoichiometry using ITC and ESI FT-ICR MS [36]. The molecular weight of Arixtra is relatively small compared to CCL7, so it was estimated that free CCL7 and the CCL7-Arixtra complex have similar ionization efficiencies. Since the total protein concentration is directly proportional to the sum of both CCL7 and CCL7-Arixtra mass spectral peak areas, the integrated peak areas of CCL7-Arixtra and CCL7 can be used to calculate their respective concentrations in solution. The association constants were calculated using Eq. 1 adapted from Wang and coworkers [38]. For each ligand concentration used, the K_a was calculated and the overall reported K_a was taken from the average measurements for the three different ligand concentrations.

Based on triplicate experiments, the association constant between CCL7-Arixtra was determined to be $1.3 \times 10^5 \text{ M}^{-1} \pm 0.1 \times 10^5 \text{ M}^{-1}$ (Table 1). For each given ligand concentration (7.5 μM , 10 μM , and 12.5 μM), the K_a was averaged to give the reported K_a shown in Table 1. Previous studies demonstrated that nano-tips pulled and sprayed under similar conditions fluctuate significantly, yielding variability in response, and poor

reproducibility [38;39]. The R value, and as a result the K_a , is sensitive to experimental conditions such as analyte concentration, pH, ionic strengths, duration of MS measurements, source conditions, and nano-tips. Thus, great care was taken to preserve conditions between experiments. Wang and coworkers found that despite fluctuations between different tips, the averaged R values for each ligand concentration were found to be similar [38]. In the present study, the R values for each concentration were obtained by averaging measurements from five identically pulled nano-ESI tips. In addition, for each nano-ESI tip used, three consecutive MS measurements were acquired under identical conditions. As in previous studies, we have also observed that association constants determined from a single nano-ESI mass spectrometry measurement are unreliable, so the use of multiple measurements carried out with at least five different tips is recommended.

The association constant measured in this study is in agreement with the binding constant derived from a solution-based method, ITC, which determined the K_a to be $2.8 \times 10^5 \text{ M}^{-1}$ [36]. Thus, this method is an accurate approach to measure the binding affinity of CCL7-Arixtra.

K_a measurements for CCL7 and differentially modified CCR2 derived peptides

Using the same titration and mass spectrometry method as described above, we determined the binding affinity of CCL7 to differentially sulfated CCR2 N-terminal peptides. Figure 3 shows a 1:1 complex formed between CCL7 and each of the CCR2 peptides used in this study. The association constant was measured by maintaining the concentration of CCL7 at $10 \text{ }\mu\text{M}$ and titrating the monosulfated or unmodified CCR2 peptides at $7.5 \text{ }\mu\text{M}$, $10 \text{ }\mu\text{M}$, and $12.5 \text{ }\mu\text{M}$. For disulfated CCR2, $5 \text{ }\mu\text{M}$, $7.5 \text{ }\mu\text{M}$, and $10 \text{ }\mu\text{M}$ were used because of the higher binding affinity between CCL7 and the peptide. Based on replicate experiments, the relative association constants between CCL7-CCR2, CCL7-monosulfated CCR2, and CCL7-disulfated CCR2 were determined to be $1.1 \times 10^4 \text{ M}^{-1}$, $3.9 \times 10^4 \text{ M}^{-1}$, and $4.0 \times 10^5 \text{ M}^{-1}$ respectively (Table 2). The binding affinity of CCL7 for CCR2 increases with the number of sulfation sites on the peptide; disulfated CCR2 has approximately a 36 fold higher binding affinity than the unmodified peptide, and monosulfated peptide has a 3.5 times higher binding affinity than the unmodified peptide.

To our knowledge this is the first reported analytical binding affinity measurement between sulfated peptides and a chemokine using mass spectrometry. It is also of note that we were able to observe the sulfated CCR2 peptide bound to CCL7 in positive ionization mode. Sulfotyrosines are characteristically labile in the positive ionization mode, but are readily observed in the negative ionization mode [40;41]. It is likely that noncovalent interactions between CCL7 and the sulfated tyrosine protects or stabilizes the sulfate modification.

The trend in binding affinities we observed between CCL7 and CCR2 is consistent with published dissociation constants for other chemokines and differentially sulfated receptor N-terminal peptides obtained using NMR. For example, CCL11 and a peptide derived from the CCR3 N-terminal region has a K_d of $140 \pm 40 \text{ }\mu\text{M}$, while CCL11 and a disulfated CCR3 complex has a K_d of less than $5 \text{ }\mu\text{M}$ [42]. Similarly, Duma *et al.* obtained a K_d of $168 \text{ }\mu\text{M}$ for CCL5 and a peptide derived from the N-terminal region of CCR5, while the K_d for CCL5 and the corresponding disulfated CCR5 peptide is less than $20 \text{ }\mu\text{M}$ [15]. In another study, the apparent dissociation constants for CXCL12-CXCR4 N-terminal peptide and CXCL12-CXCR4 trisulfated peptide was determined to be $4.5 \pm 2.2 \text{ }\mu\text{M}$ and $0.2 \pm 0.2 \text{ }\mu\text{M}$, respectively [10;14]. Together, these studies suggest that the role of sulfation in chemokine receptors may be to enhance interactions with chemokine ligands.

Competitive binding of CCR2 and GAG with CCL7

Using mass spectrometry, we did not observe significant ternary complex formation from solutions of equal concentrations of CCL7, Arixtra and disulfated CCR2 peptide. Therefore, competitive binding conditions were used to investigate the binding relationship between CCL7 and its two ligands. Mass spectra from mixtures of CCL7, Arixtra, and disulfated CCR2 21-30 at various ratios show that Arixtra has a lower affinity than the CCR2 peptide for CCL7. Figure 4 shows the mass spectra at concentrations of 10 μ M for each of CCL7 and Arixtra, and the concentration of disulfated CCR2 is 1 μ M in the upper spectrum and 10 μ M in the lower spectrum. Peaks representing both CCL7-Arixtra and CCL7-disulfated CCR2 were observed. The intensity of CCL7-Arixtra was decreased by 60% upon an increase of disulfated CCR2 from 0 μ M to 10 μ M, indicating that Arixtra had been partly displaced by disulfated CCR2 21-30.

A mass spectrometry titration plot was used to monitor the amounts of chemokine-GAG and chemokine-peptide with increasing concentrations of peptide. The normalized peak integrations of the free CCL7, CCL7-Arixtra, and CCL7- disulfated CCR2 were plotted against the concentration of disulfated CCR2. To obtain normalized values for each given species at the various concentrations noted, the peak areas corresponding to each species were divided by the sum of integrations for all species observed. Figure 5 represents a summary of the data collected from mass spectra of samples containing 10 μ M CCL7, 10 μ M Arixtra, and varying amounts of disulfated CCR2 (0–10 μ M). Increasing the concentration of disulfated CCR2 resulted in a ratio shift of CCL7-Arixtra to CCL7-CCR2 complex in which the formation of more CCL7-CCR2 complex coincided with a decrease of CCL7-Arixtra complex. This is highly suggestive of competitive binding of CCL7 with disulfated CCR2 peptide and Arixtra. When the three analytes are sprayed at equal concentrations (10 μ M), the mass spectra peaks representing CCL7-disulfated CCR2 are noticeably more abundant than those peaks representing CCL7-Arixtra. Hence, competitive assay shows that disulfated CCR2 21-30 has a relatively higher binding affinity than Arixtra, which is expected since association constant for CCL7-disulfated CCR2 was determined to be three fold greater than that of CCL7-Arixtra.

Observations that suggest CCR2 competes with GAGs for occupation of CCL7 binding sites are consistent with mutation studies with chemokine CCL2 that indicate there is partial overlap between the GAG and CCR2 binding domains [43;44;45]. Therefore, it is unlikely that monomeric CCL2 will be able to bind to GAGs and its receptor concurrently. Since CCL7 has a 74% sequence identity with CCL2, it is probable that CCL7 also has partially overlapping GAG and CCR2 binding domains. Also, a non-binding GAG CCL7 mutant showed minor perturbations in its affinity for CCR2 [46], supporting the hypothesis that the binding sites may partially overlap. In this study, it was demonstrated that a ternary complex does not form in the gas phase, and binding of Arixtra and CCR2 for CCL7 is partially competitive. Additional *in vivo* studies are needed to conclusively determine the nature of the relationship between CCL7, GAG and CCR2.

Conclusions

In this study, we have applied a mass spectrometry method to measure the association constants for chemokine and sulfated receptor peptides. This method allows for accurate K_a measurements that agree well with values obtained using traditional solution methods. Thus, nano-ESI MS can be applied to determine the contribution of tyrosine sulfation to the binding affinity of other proteins and sulfated peptides. The benefits of this nano-ESI MS approach include minimal sample preparation, low sample consumption, and rapid data analysis. In addition, nano-ESI TOF MS can be used to study competitive binding

interactions between multiple biomolecules to provide insights into the nature of their binding interactions.

Acknowledgments

This work was supported in part by National Institutes of Health (NIH) Grant HD056022 and by GM47356

References

1. Handel TM, Johnson Z, Crown SE, Lau EK, Proudfoot AE. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annu Rev Biochem* 2005;74:385–410. [PubMed: 15952892]
2. Parish CR. The role of heparan sulphate in inflammation. *Nat Rev Immunol* 2006;6:633–643. [PubMed: 16917509]
3. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;7:678–689. [PubMed: 17717539]
4. Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* 2007;25:787–820. [PubMed: 17291188]
5. Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, Borlat F, Wells TN, Kosco-Vilbois MH. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* 2003;100:1885–1890. [PubMed: 12571364]
6. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, Gerard C, Sodroski J, Choe H. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell* 1999;96:667–676. [PubMed: 10089882]
7. Farzan M, Vasilieva N, Schnitzler CE, Chung S, Robinson J, Gerard NP, Gerard C, Choe H, Sodroski J. A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *J Biol Chem* 2000;275:33516–33521. [PubMed: 10938094]
8. Bannert N, Craig S, Farzan M, Sogah D, Santo NV, Choe H, Sodroski J. Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J Exp Med* 2001;194:1661–1673. [PubMed: 11733580]
9. Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, Mirzabekov T, Choe H. The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and HIV-1 entry. *J Biol Chem* 2002;277:29484–29489. [PubMed: 12034737]
10. Veldkamp CT, Seibert C, Peterson FC, Sakmar TP, Volkman BF. Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12). *J Mol Biol* 2006;359:1400–1409. [PubMed: 16725153]
11. Preobrazhensky AA, Dragan S, Kawano T, Gavrillin MA, Gulina IV, Chakravarty L, Kolattukudy PE. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. *J Immunol* 2000;165:5295–5303. [PubMed: 11046064]
12. Gutierrez J, Kremer L, Zaballos A, Goya I, Martinez AC, Marquez G. Analysis of post-translational CCR8 modifications and their influence on receptor activity. *J Biol Chem* 2004;279:14726–14733. [PubMed: 14736884]
13. Colvin RA, Campanella GS, Manice LA, Luster AD. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Mol Cell Biol* 2006;26:5838–5849. [PubMed: 16847335]
14. Seibert C, Veldkamp CT, Peterson FC, Chait BT, Volkman BF, Sakmar TP. Sequential tyrosine sulfation of CXCR4 by tyrosylprotein sulfotransferases. *Biochemistry* 2008;47:11251–11262. [PubMed: 18834145]
15. Duma L, Haussinger D, Rogowski M, Lusso P, Grzesiek S. Recognition of RANTES by extracellular parts of the CCR5 receptor. *J Mol Biol* 2007;365:1063–1075. [PubMed: 17101151]

16. Simpson LS, Zhu JZ, Widlanski TS, Stone MJ. Regulation of chemokine recognition by site-specific tyrosine sulfation of receptor peptides. *Chem Biol* 2009;16:153–161. [PubMed: 19246006]
17. Loo JA. Studying noncovalent protein complexes by electrospray ionization mass spectrometry. *Mass Spectrom Rev* 1997;16:1–23. [PubMed: 9414489]
18. Leavitt S, Freire E. Direct measurement of protein binding energetics by isothermal titration calorimetry. *Curr Opin Struct Biol* 2001;11:560–566. [PubMed: 11785756]
19. Wilm M, Mann M. Analytical properties of the nanoelectrospray ion source. *Anal Chem* 1996;68:1–8. [PubMed: 8779426]
20. Karas M, Bahr U, Dulcks T. Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine. *Fresenius J Anal Chem* 2000;366:669–676. [PubMed: 11225778]
21. Hsieh FY, Tong X, Wachs T, Ganem B, Henion J. Kinetic monitoring of enzymatic reactions in real time by quantitative high-performance liquid chromatography-mass spectrometry. *Anal Biochem* 1995;229:20–25. [PubMed: 8533890]
22. Gao Q, Cheng X, Smith RD, Yang CF, Goldberg IH. Binding specificity of post-activated neocarzinostatin chromophore drug-bulged DNA complex studied using electrospray ionization mass spectrometry. *J Mass Spectrom* 1996;31:31–36. [PubMed: 8799259]
23. Sannes-Lowery KA, Hu P, Mack DP, Mei HY, Loo JA. HIV-1 Tat peptide binding to TAR RNA by electrospray ionization mass spectrometry. *Anal Chem* 1997;69:5130–5135. [PubMed: 9414617]
24. Lim HK, Hsieh YL, Ganem B, Henion J. Recognition of Cell-Wall Peptide Ligands by Vancomycin Group Antibiotics - Studies Using Ion-Spray Mass-Spectrometry. *Journal of Mass Spectrometry* 1995;30:708–714.
25. Jorgensen TJD, Roepstorff P, Heck AJR. Direct determination of solution binding constants for noncovalent complexes between bacterial cell wall peptide analogues and vancomycin group antibiotics by electrospray ionization mass spectrometry. *Analytical Chemistry* 1998;70:4427–4432.
26. Peschke M, Verkerk UH, Kebarle P. Features of the ESI mechanism that affect the observation of multiply charged noncovalent protein complexes and the determination of the association constant by the titration method. *Journal of the American Society for Mass Spectrometry* 2004;15:1424–1434. [PubMed: 15465355]
27. Loo JA, Hu PF, McConnell P, Mueller WT, Sawyer TK, Thanabal V. A study of Src SH2 domain protein-phosphopeptide binding interactions by electrospray ionization mass spectrometry. *Journal of the American Society for Mass Spectrometry* 1997;8:234–243.
28. Robinson CV, Chung EW, Kragelund BB, Knudsen J, Aplin RT, Poulsen FM, Dobson CM. Probing the nature of noncovalent interactions by mass spectrometry. A study of protein-CoA ligand binding and assembly. *Journal of the American Chemical Society* 1996;118:8646–8653.
29. Wu QY, Gao JM, JosephMcCarthy D, Sigal GB, Bruce JE, Whitesides GM, Smith RD. Carbonic anhydrase-inhibitor binding: From solution to the gas phase. *Journal of the American Chemical Society* 1997;119:1157–1158.
30. Schenauer MR, Leary JA. An Ion Mobility-Mass Spectrometry Investigation of Monocyte Chemoattractant Protein-1. *International Journal of Mass Spectrometry* 2009;287:70–76. [PubMed: 20160907]
31. Benkestock K, Sundqvist G, Edlund PO, Roeraade J. Influence of droplet size, capillary-cone distance and selected instrumental parameters for the analysis of noncovalent protein-ligand complexes by nano-electrospray ionization mass spectrometry. *J Mass Spectrom* 2004;39:1059–1067. [PubMed: 15386746]
32. Sobott F, Robinson CV. Protein complexes gain momentum. *Curr Opin Struct Biol* 2002;12:729–734. [PubMed: 12504676]
33. Wang W, Kitova EN, Klassen JS. Influence of solution and gas phase processes on protein-carbohydrate binding affinities determined by nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem* 2003;75:4945–4955. [PubMed: 14708765]

34. Kuschert GS, Coulin F, Power CA, Proudfoot AE, Hubbard RE, Hoogewerf AJ, Wells TN. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry* 1999;38:12959–12968. [PubMed: 10504268]
35. Yu Y, Sweeney MD, Saad OM, Crown SE, Hsu AR, Handel TM, Leary JA. Chemokine-glycosaminoglycan binding: specificity for CCR2 ligand binding to highly sulfated oligosaccharides using FTICR mass spectrometry. *J Biol Chem* 2005;280:32200–32208. [PubMed: 16033763]
36. Yu Y, Sweeney MD, Saad OM, Leary JA. Potential inhibitors of chemokine function: analysis of noncovalent complexes of CC chemokine and small polyanionic molecules by ESI FT-ICR mass spectrometry. *J Am Soc Mass Spectrom* 2006;17:524–535. [PubMed: 16503157]
37. Yu G, LeBrun L, Gunay NS, Hoppensteadt D, Walenga JM, Fareed J, Linhardt RJ. Heparinase I acts on a synthetic heparin pentasaccharide corresponding to the antithrombin III binding site. *Thromb Res* 2000;100:549–556. [PubMed: 11152935]
38. Wang W, Kitova EN, Klassen JS. Determination of protein-oligosaccharide binding by nanoelectrospray fourier-transform ion cyclotron resonance mass spectrometry. *Methods Enzymol* 2003;362:376–397. [PubMed: 12968378]
39. Gabelica V, Vreuls C, Filee P, Duval V, Joris B, Pauw ED. Advantages and drawbacks of nanospray for studying noncovalent protein-DNA complexes by mass spectrometry. *Rapid Commun Mass Spectrom* 2002;16:1723–1728. [PubMed: 12207359]
40. Onnerfjord P, Heathfield TF, Heinegard D. Identification of tyrosine sulfation in extracellular leucine-rich repeat proteins using mass spectrometry. *J Biol Chem* 2004;279:26–33. [PubMed: 14551184]
41. Salek M, Costagliola S, Lehmann WD. Protein tyrosine-O-sulfation analysis by exhaustive product ion scanning with minimum collision offset in a NanoESI Q-TOF tandem mass spectrometer. *Anal Chem* 2004;76:5136–5142. [PubMed: 15373453]
42. Stone MJ, Chuang S, Hou X, Shoham M, Zhu JZ. Tyrosine sulfation: an increasingly recognised post-translational modification of secreted proteins. *N Biotechnol* 2009;25:299–317. [PubMed: 19658209]
43. Lau EK, Paavola CD, Johnson Z, Gaudry JP, Geretti E, Borlat F, Kungl AJ, Proudfoot AE, Handel TM. Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1: implications for structure and function in vivo. *J Biol Chem* 2004;279:22294–22305. [PubMed: 15033992]
44. Hemmerich S, Paavola C, Bloom A, Bhakta S, Freedman R, Grunberger D, Krstenansky J, Lee S, McCarley D, Mulkins M, Wong B, Pease J, Mizoue L, Mirzadegan T, Polsky I, Thompson K, Handel TM, Jarnagin K. Identification of residues in the monocyte chemotactic protein-1 that contact the MCP-1 receptor, CCR2. *Biochemistry* 1999;38:13013–13025. [PubMed: 10529171]
45. Jarnagin K, Grunberger D, Mulkins M, Wong B, Hemmerich S, Paavola C, Bloom A, Bhakta S, Diehl F, Freedman R, McCarley D, Polsky I, Ping-Tsou A, Kosaka A, Handel TM. Identification of surface residues of the monocyte chemotactic protein 1 that affect signaling through the receptor CCR2. *Biochemistry* 1999;38:16167–16177. [PubMed: 10587439]
46. Ali S, Robertson H, Wain JH, Isaacs JD, Malik G, Kirby JA. A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *J Immunol* 2005;175:1257–1266. [PubMed: 16002730]

A **B**

Figure 1.
Structures of (A) Arixtra and (B) disulfated CCR2 21-30 peptide.

Figure 2.
Nano-ESI mass spectrum of 10 μ M of CCL7 and 10 μ M of Arixtra sprayed from 125 mM NH_4OAc , pH 6.8.

Figure 3.

Mass spectra of CCL7 and differentially sulfated CCR2 N-terminal peptides obtained in 125 mM ammonium acetate, pH 6.8. Shown are mass spectra of 10 μ M CCL7 and (A) 10 μ M CCR2, (B) 10 μ M monosulfated CCR2, (C) 10 μ M disulfated CCR2. CCR2 is observed in the mass spectrum of CCL7 and monosulfated CCR2 (B) because the sulfoester bond is labile and loss of SO_3 occurs.

Figure 4.

Mass spectra of CCL7 and Arixtra with the addition of competing ligand, disulfated CCR2 21-30. The upper spectrum (A) represents a sample containing 10 μ M CCL7, 10 μ M Arixtra, and 1 μ M disulfated CCR2. The lower spectrum (B) represents a sample containing 10 μ M CCL7, 10 μ M Arixtra, and 10 μ M disulfated CCR2.

Figure 5.

MS titration plot monitoring the percentage of free CCL7, CCL7-Arixtra, and CCL7-disulfated CCR2 21-30 complex with increasing the concentration of peptide from 0 to 10 μM. The concentrations of CCL7 and Arixtra were kept constants at 10 μM.

Table 1

Comparison of association constants of CCL7-Arixtra

methods	Ka (M ⁻¹)
nanoESI-MS	$1.3 \times 10^5 \pm 0.1 \times 10^5$ *
Isothermal titration calorimetry	$2.8 \times 10^5 \pm 0.8 \times 10^5$

* the values represent the average $\pm$ standard deviation of three measurements

Table 2Association constants (K_a) of CCL7 and differentially sulfated CCR2 21-30

ligand	concentration of peptide (μM)	K_a (M^{-1})	avg K_a (M^{-1})
CCR2 21-30	7.5	1.2×10^4	$1.1 \times 10^4 \pm 0.1 \times 10^4^*$
	10	1.0×10^4	
	12.5	0.99×10^4	
monosulfated	7.5	3.7×10^4	$3.9 \times 10^4 \pm 0.2 \times 10^4^*$
CCR2 21-30	10	4.0×10^4	
	12.5	4.0×10^4	
	5	4.1×10^5	$4.0 \times 10^5 \pm 0.1 \times 10^5^*$
disulfated	7.5	4.1×10^5	
CCR2 21-30	10	3.9×10^5	

*
the error values represent the standard deviation