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Sulfated oligosaccharides (heparin and fucoidan) binding and dimerization of stromal cell-derived factor-1 (SDF-1/CXCL 12) are coupled as evidenced by affinity CE-MS analysis

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Chemokine stromal cell-derived factor-1 (SDF-1) is a potent chemoattractant involved in leukocyte trafficking and metastasis. Heparan sulfate on the cell surface binds SDF-1 and may modulate its function as a coreceptor of this chemokine. A major effect of the glycosaminoglycan binding may be on the quaternary structure of SDF-1, which has been controversially reported as a monomer or a dimer. We have investigated the effect of sulfated oligosaccharides on the oligomerization of SDF-1 and of its mutated form SDF-1 (3/6), using affinity capillary electrophoresis (ACE) hyphenated to mass spectrometry (MS). Coupled to MS, ACE allowed the study for the first time of the effect of size-defined oligosaccharides on the quaternary organization of SDF-1 in μM range concentrations, i.e., lower values than the mM values previously reported in NMR, light scattering, and ultracentrifugation experiments. Our results showed that in the absence of sulfated oligosaccharides, SDF-1 is mostly monomeric in solution. However, dimer formation was observed upon interaction with heparin-sulfated oligosaccharides despite the mM Kd values for dimerization. A SDF-1/oligosaccharide 2/1 complex was detected, indicating that oligosaccharide binding promoted the dimerization of SDF-1. Heparin tetrasaccharide but not disaccharide promoted dimer formation, suggesting that the dimer required to be stabilized by a long enough bound oligosaccharide. The SDF-1/oligosaccharide 1/1 complex was only observed with heparin disaccharide and fucoidan pentasaccharide, pointing out the role of specific structural determinants in promoting dimer formation. These results underline the importance of dimerization induced by glycosaminoglycans for chemokine functionality.

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Introduction

The protein mediator stromal cell-derived factor-1 (SDF-1/CXCL 12) belonging to the chemokine family is a potent chemoattractant involved in leukocyte trafficking, inflammatory response, and cancer metastasis (Crump et al. 1997; Muller et al. 2001). SDF-1 also plays important roles in hematopoiesis and in developmental processes as shown by gene knockout experiments (Tachibana et al. 1998). Defective SDF-1 leads to lethal phenotype and alterations in B-cell lymphopoiesis and bone-marrow myelopoiesis (Nagasawa et al. 1996). These biological functions are mediated by the binding of SDF-1 to the G-protein coupled receptor, CXCR4, which is known to be the major receptor for this chemokine (Bleul et al. 1996). Prior to binding to CXCR4, SDF-1 is first recruited from the fluid phase through interactions with extracellular matrix and cell surface glycosaminoglycans (GAGs) (Mbemba et al. 2000). The in vitro binding of SDF-1 to heparin, a GAG analog of the cell surface heparan sulfate (HS), had been demonstrated by affinity chromatography using a heparin column and by surface plasmon resonance (Amara et al. 1999). More recently, SDF-1 was shown to bind to the HS proteoglycan syndecan-4 at the surface of HeLa cells in culture and to form complexes comprising syndecan-4 and CXCR4 (Hamon et al. 2004). Furthermore, it has been shown that CXCR4 serves as a receptor on T cells for X4 HIV strain and that virus entry could be inhibited by SDF-1 in an optimal manner in the presence of cell surface HS (Valenzuela-Fernandez et al. 2001). All together, these results suggest the concomitant binding of SDF-1 to CXCR4 and HS. Given these properties, the GAG heparan sulfate can be considered as a coreceptor of SDF-1, leading to the proposal that GAG/chemokine interactions may define new targets for disease therapy (Cascieri and Springer 2000; Johnson et al. 2005). Supporting this hypothesis, the recent studies reported that soluble heparin and heparan sulfate inhibited SDF-1-mediated chemotaxis in vitro (Murphy et al. 2007). In addition, preincubation of SDF-1 with heparin significantly affected growth, migration, and invasion of hepatoma cells (Sutton et al. 2007).

The role of interaction of SDF-1 with cell surface GAGs in the biological activity of SDF-1 remains to be deciphered, though several data reported for SDF-1 and other chemokines suggest that it may provide a mechanism for the modulation of chemokine signaling (Proudfoot et al. 2003). A consequence of sequestration of the chemokine from the fluid phase by cell surface GAGs is a localized increase of its concentration at the membrane surface, paving a chemokine gradient for the cells moving to the inflammatory site. An additional major effect of

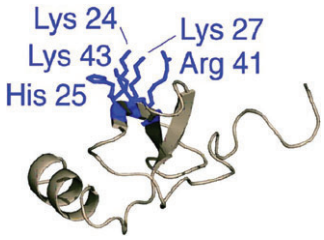


Fig. 1. Structural representation of SDF-1 showing its characteristic tertiary fold and the residues involved in the binding of sulfated glycosaminoglycans (Handel et al. 2005).

the locally high chemokine concentration may be on its quaternary structures and oligomerization status. SDF-1 adopts the well-characterized tertiary fold conserved among the chemokine family, which consists of a flexible N-terminus followed by a triple-stranded antiparallel β-sheet overlaid by a C-terminal α-helix (Figure 1). The protein surface analysis and energy calculation of a set of chemokines including SDF-1 indicated that this monomeric template is well suited for dimerization (Lortat-Jacob et al. 2002). The X-ray crystallography and NMR structural studies of several chemokines showed dimerized structures, thus confirming initially this oligomerization property. However, the high chemokine concentration and the buffer conditions required by these structural studies call into question the physiological relevance of the observed dimers. Furthermore, dissociation constants determined for certain of them were most often in the mM range, thus well above their in vivo concentration.

As regards SDF-1, contradictory data have been reported about the quaternary structure adopted by this chemokine, so that its functional form, either monomeric or dimeric, is controversial. While the reported X-ray structures revealed a dimer (Dealwis et al. 1998; Ohnishi et al. 2000), NMR analyses, which were all conducted in mM range concentrations, indicated either a monomeric form (Crump et al. 1997) or a weak dimeric association with mM Kd values (Holmes et al. 2001; Gozansky et al. 2005). A Kd of 5 mM was recently determined based on relaxation data from a ^{15}N NMR dynamic study of SDF-1, corresponding to 27.4% of dimer in the most concentrated sample (2.6 mM) (Baryshnikova and Sykes 2006). A recent NMR study pointed out the dependence of the monomer–dimer equilibrium of SDF-1 on the buffer composition (Veldkamp et al. 2005). The dimer formation is favored by high ionic strength, nonacid pH values, phosphate buffer, and the presence of negative multivalent anions such as sulfate.

Given the Kd values reported in vitro and the nM physiological concentration of SDF-1, we may not expect dimerization in vivo. Nevertheless, current models propose that oligomerization of the chemokine may still occur in vivo and be functionally important, through a promoting role of the cell surface HS. Binding to HS and the subsequent increase of the local concentration of the chemokine may promote its dimerization, and thereby affect its presentation to the receptor. However, no direct evidence of SDF-1 dimerization at a concentration closer to physiological conditions and of the role of sulfated carbohydrates in this process has been provided to date.

Because of the role of SDF-1 in normal and pathological processes, we have investigated the dimerization of SDF-1 in μM concentration and in the presence of sulfated oligosaccharides, using affinity capillary electrophoresis (ACE) hyphenated to electrospray ionization-mass spectrometry (ESI/MS). ACE-ESI/MS coupling is a novel analytical strategy that we have recently introduced for the characterization of protein/carbohydrate complexes such as antithrombin/heparin pentasaccharide (Fermas et al. 2007). It is based on the ability of capillary electrophoresis (CE) to separate the free and complexed protein species and to be coupled to MS for the online characterization of the noncovalent complex in nondenaturing conditions. A major advantage of biomolecular CE is that it allows interactions freely in solution, with no need for immobilization of the interacting molecules. Furthermore, hyphenation of CE with ESI/MS allows the direct characterization of complex formation and online structural determination of the oligosaccharide ligand without the need for additional isolation and purification steps. In the study herein, we have shown that the highly basic protein SDF-1 ($pI = 9.9$) can be detected by CE-ESI/MS. We have studied its capability of dimerization and of forming noncovalent complexes upon interaction with sulfated oligosaccharides derived from heparin and fucoidan. To date, the interaction of a SDF-1 dimer has been reported by X-ray crystallography and NMR only with a heparin disaccharide (disaccharide I-S) (Murphy et al. 2007), a structure too small to account for the in vivo interaction with cell surface HS. Here, we report on the interaction of SDF-1 with di-, tetra-, and hexasaccharides derived from heparin, a HS analog. For the sake of comparison, interaction experiments were also performed with a synthetic pentasaccharide reproducing the structure repeat found in fucoidan (Chevolot et al. 2001; Hua et al. 2004; Daniel et al. 2007). Fucoidan is an algal sulfated polysaccharide having anticoagulant, antithrombotic (Mourao 2004), and antiproliferative effects in mammalian systems (Logeart et al. 1997) and exhibiting interesting biological activities in inflammation and toward immune systems through interactions with target proteins, among them the complement proteins and cytokines (Nika et al. 2003; Tissot et al. 2003, 2005). Fucoidan has been shown to elevate white blood cells and mobilize hematopoietic progenitor/stem cells in mice and primates, due to disruption of the SDF-1 gradient between bone marrow and peripheral blood (Sweeney et al. 2002). We show by CE-ESI/MS for the first time, the effect of these GAG analogs on the dimerization of SDF-1. Significant differences were observed between heparin and fucoidan oligosaccharides in their ability to promote SDF-1 dimerization, clarifying the specific role of structural determinants of the carbohydrate molecule. The SDF-1 dimer noncovalently associated with an oligosaccharide chain was detected, and the mass determination of the noncovalent complex led to the binding stoichiometry. The capability of dimerization of the site-directed mutant SDF-1 (3/6) known to have lost its binding capacity to HS and heparin was also investigated.

Results

Characterization of SDF-1 and SDF-1 (3/6) by capillary electrophoresis-ESI/MS

The purpose of the study herein was to investigate the noncovalent complexes formed by the chemokine SDF-1 upon contact with sulfated oligosaccharides using capillary electrophoresis

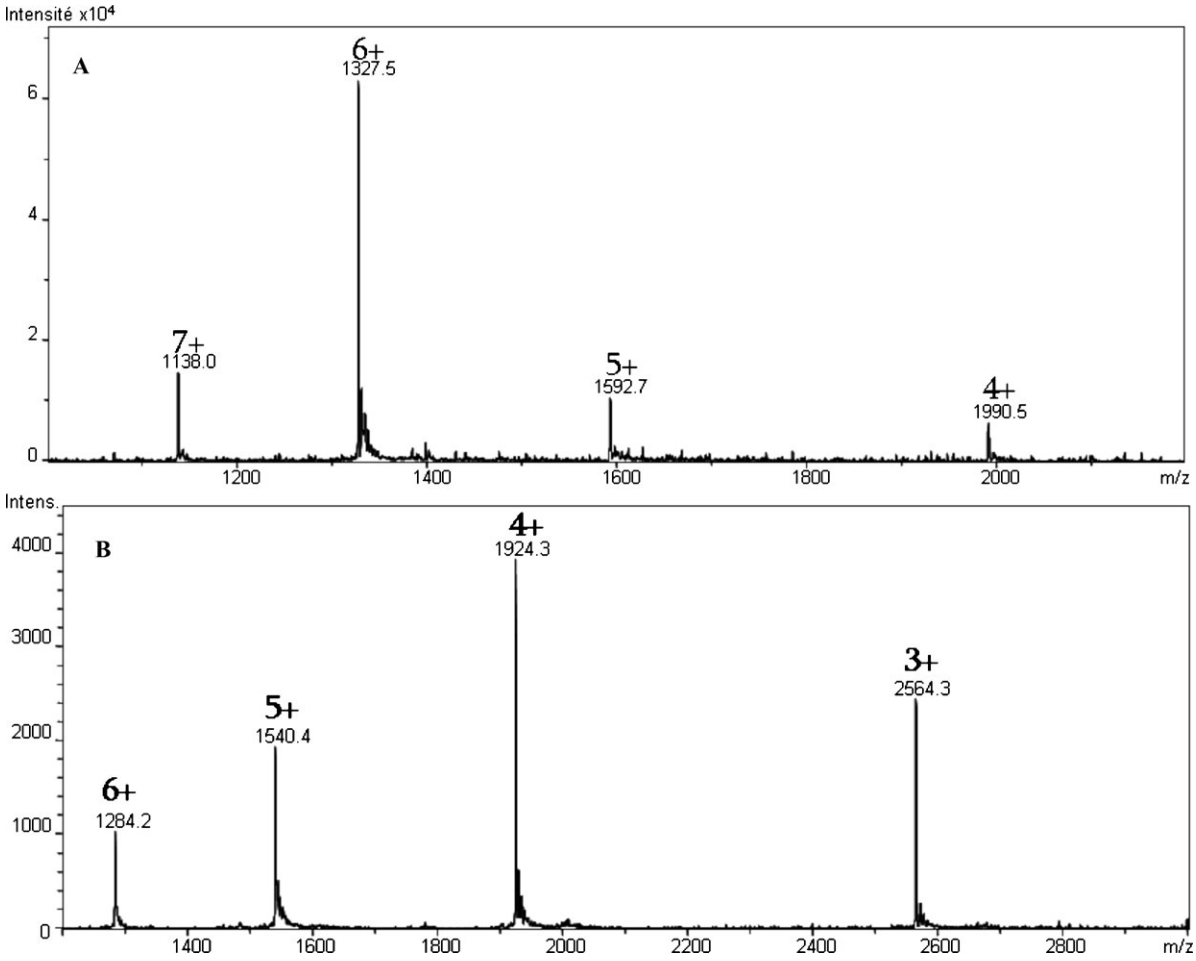


Fig. 2. Positive ESI mass spectra of (**A**) SDF-1 and (**B**) SDF-1 (3/6) acquired by CE-MS. CE conditions: PEO-coated fused silica capillary, 50 μm i.d., capillary length: 98 cm (21.5 cm to detector); separation electrolyte: 75 mM ammonium acetate, pH 6.5; the chemokines (15 μM) were injected at 50 mbar for 30 s; separation voltage: +10 kV; temperature: 25°C; continuous hydrodynamic pressure: 50 mbar. MS conditions: target mass: 3000 *m/z*. Sheath liquid: water/methanol (90:10 v/v), flow rate: 3 μL min^{-1}; Nebulization gas: 5 psi; drying gas: 3 L min^{-1}, 100°C.

coupled to ESI/MS. We previously established that this basic protein ($pI = 9.9$) was amenable to CE analysis (data not shown) using a dynamic PEO coating of the capillary (Fermas et al. 2008). In order to detect noncovalent complexes by MS, this CE separation was hyphenated to mass spectrometry using positive ESI and a 10% methanol aqueous sheath liquid. Under these conditions allowing a soft desolvation, both proteins SDF-1 and SDF-1 (3/6) (15 μM) were easily detected as shown in the mass spectra displayed in Figure 2. The spectrum of SDF-1 (Figure 2A) showed four ions with a charge distribution from +7 (*m/z* 1138.0) to +4 (*m/z* 1990.5) corresponding to a molecular mass of 7958.5 ± 0.5 g mol^{-1}, in agreement with the theoretical average molecular mass of 7959.4 g mol^{-1}. The spectrum obtained for SDF-1 (3/6) (Figure 2B) showed four ions with a charge distribution from +6 (*m/z* 1284.2) to +3 (*m/z* 2564.3) corresponding to a molecular mass of 7694.8 ± 4.1 g mol^{-1}, in agreement with the theoretical average molecular mass of 7699.0 g mol^{-1}. The charge state distribution observed for SDF-1 (3/6) was shifted toward lower values and the intensity of the detected ions was weaker than in the mass spectrum of SDF-1 (Figure 2A), likely due to the replacement of three basic residues by three neutral Ser residues in the mutated chemokine. These features of SDF-1 (3/6) were also in agreement with its lower electrophoretic mobility ($\mu_{ep,\ SDF\text{-}1(3/6)} = 13.9 \times 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$) compared to SDF-1 ($\mu_{ep,\ SDF\text{-}1} = 21 \times 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$) (data not shown) accordingly to its lower *pI* (9.56). It is worth noting that we did not observe ions corresponding to dimers, indicating that SDF-1 is in monomeric form at the concentration used (15 μM).

Study of the interaction of SDF-1 with heparin and fucoidan oligosaccharides by ACE-ESI/MS

We previously reported that ACE is a well-suited method for the characterization of noncovalent complexes between a protein and a carbohydrate ligand through a shift of the protein peak according to the concentration of ligand in the electrolyte (Varenne et al. 2003). The interaction of SDF-1 with size-defined heparin oligosaccharides ranging from di- to hexasaccharides was studied by ACE hyphenated to ESI/MS (ACE-MS) using the coupling conditions described above. ACE-MS with 15 μM disaccharide I-S (α-ΔUA-2S-[1→4]-GlcNS-6S) in the electrolyte led to a mass spectrum exhibiting two charge distributions. A focus on the charge state +4 region (Figure 3A) shows, in

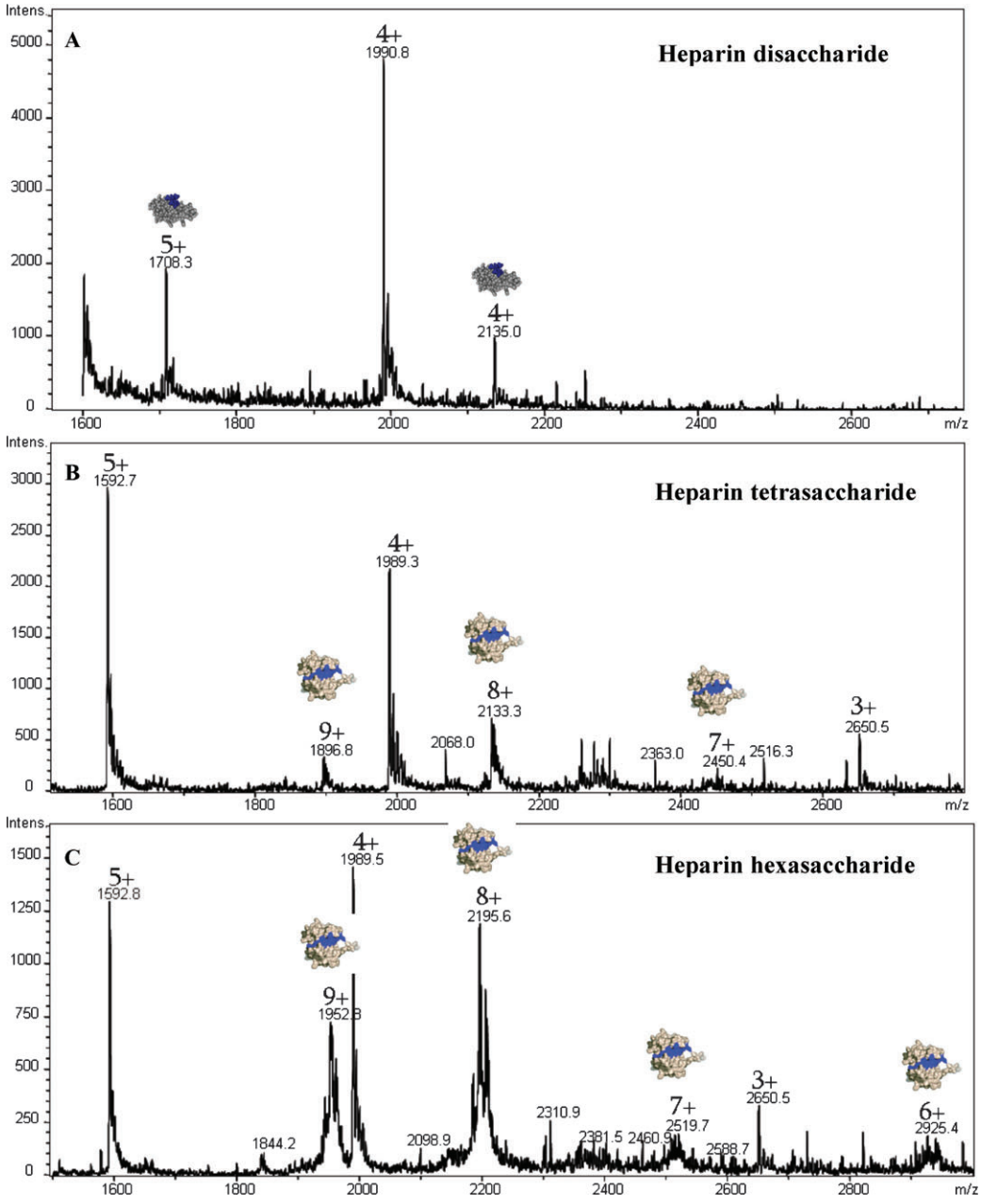


Fig. 3. Positive ESI mass spectra of SDF-1 upon interaction with heparin oligosaccharides acquired by affinity capillary electrophoresis-mass spectrometry (ACE-MS). Affinity capillary electrophoresis of SDF-1 was carried out in the presence of heparin oligosaccharide in the electrolyte (**A**) with 15 μM heparin disaccharide I-S, (**B**) with 3 μM heparin tetrasaccharide, and (**C**) with 1 μM heparin hexasaccharide. SDF-1 monomer and dimer structures represented above the corresponding ions are from (Handel et al. 2005). CE conditions: PEO-coated fused silica capillary, 50 μm i.d., capillary length: 98 cm (21.5 cm to detector); separation electrolyte: 75 mM ammonium acetate, pH 6.5; SDF-1 (15 μM) was injected at 50 mbar for 60 s; separation voltage: +10 kV; temperature: 25°C; continuous hydrodynamic pressure: 50 mbar. MS conditions: target mass: 3000 *m/z*. Sheath liquid: water/methanol (90:10 v/v), flow rate: 3 μL min^{-1}; Nebulization gas: 5 psi; drying gas: 3 L min^{-1}, 100°C.

addition to the monomeric SDF-1 species (charge state +4 at *m/z* 1990.8, corresponding to an experimental molecular mass of SDF-1 of 7959.2), a species represented by the two ions at *m/z* 1708.3 (charge state +5) and *m/z* 2135.0 (charge state +4) corresponding to the molecular mass of 8536.2 ± 0.4 g mol^{-1}. This value matches with the sum of the masses of one molecule of SDF-1 and one molecule of disaccharide (experimental molecular mass 577.0 g mol^{-1}), suggesting that the detected species corresponded to a 1/1 SDF-1/sulfated disaccharide complex. No ion corresponding to the dimeric form of SDF-1 was detected, while a dimeric structure of SDF-1 cocrystallized with disaccharide I-S has been reported in the X-ray structure (Murphy et al. 2007).

ACE-MS experiments with longer heparin oligosaccharides such as sulfated tetrasaccharide and hexasaccharide resulted in the mass spectra showing, in addition to monomeric SDF-1 (charge states from +5 to +3, $M = 7953.4 \pm 5.0$ g mol^{-1}), new species of higher charge states ranging from +9 to +6. With

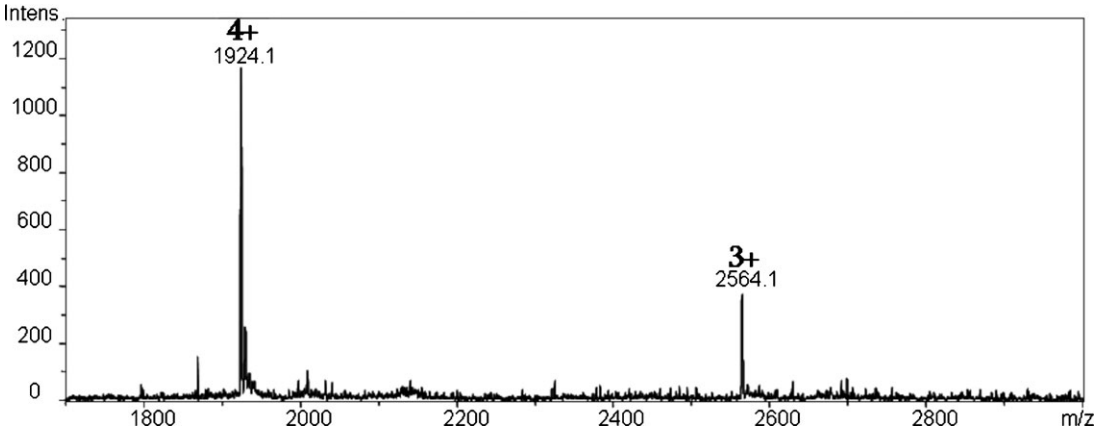


Fig. 4. Positive ESI mass spectrum of SDF-1 (3/6) upon interaction with heparin hexasaccharide acquired by affinity capillary electrophoresis-mass spectrometry (ACE-MS). Affinity capillary electrophoresis of SDF-1 (3/6) (15 μM) was carried out in the presence of 3 μM heparin hexasaccharide in the electrolyte. CE conditions: same as in Figure 3.

heparin tetrasaccharide (3 μM) in the electrolyte, this new species appeared as three ions with a charge distribution from +9 (*m/z* 1896.8) to +7 (*m/z* 2436.2) corresponding to the molecular mass of 17055.4 ± 8.1 g mol^{-1} (Figure 3B). This value corresponded to the molecular mass of a dimer of SDF-1 complexed with one molecule of tetrasaccharide. With heparin hexasaccharide used at 1 μM in the electrolyte, the same 2/1 complex comprising two SDF-1 molecules and one hexasaccharide could be inferred from the four ions with a charge distribution from +9 (*m/z* 1952.8) to +6 (*m/z* 2925.4) and corresponding to the molecular mass of 17556.9 ± 8.1 g mol^{-1} (Figure 3C). Except for the 2/1 SDF-1/oligosaccharide complex, no other noncovalent complex was detected with these oligosaccharides, particularly such as a 1/1 complex as observed for the disaccharide. It is worth noting that the SDF-1 dimer is observed only in association with one oligosaccharide molecule and that no free dimer is detected. Given the lowest dissociation constants previously reported for the SDF-1 dimer (120–170 μM) (Holmes et al. 2001; Veldkamp et al. 2005) and the chemokine concentration (15 μM) used in these ACE-MS experiments, SDF-1 should not dimerize. Hence, the detection of the SDF-1 dimer associated with an oligosaccharide chain pointed out the role of the sulfated carbohydrate in promoting dimerization and in the stabilization of the noncovalent complex. The relative and absolute intensities of ions corresponding to the 2/1 complex were enhanced from tetrasaccharide to hexasaccharide, despite the lower concentration of hexasaccharide, suggesting a higher stability of the complex with a longer oligosaccharide chain.

When using the modified chemokine SDF-1 (3/6), we were unable to find evidence for the dimer or complex with any oligosaccharide in ACE-MS experiments. The mass spectrum shown in Figure 4 and corresponding to ACE-MS experiment with 3 μM heparin hexasaccharide in electrolyte showed ions with charge states +4 (*m/z* 1924.1) and +3 (*m/z* 2564.1) characteristic of monomeric SDF-1 (3/6). No complex with the hexasaccharide was observed despite the higher concentration of hexasaccharide (3 μM) used with SDF-1 (3/6) than with SDF-1 (1 μM). This feature was confirmed by ACE experiments (not shown) in which heparin hexasaccharide was added at increasing concentrations in the electrolyte buffer. While hexasaccharide at 0.25 and 0.5 μM resulted in a decrease of SDF-1 mobility to $\mu_{ep,\ SDF\text{-}1} = 15 \times 10^{-5}$ cm^2 V^{-1} s^{-1} and 2×10^{-5}cm^2 V^{-1} s^{-1}, respectively, indicating a strong interaction between SDF-1 and the heparin hexasaccharide; the electrophoretic mobility of SDF-1 (3/6) was almost unchanged under these conditions. A cluster of basic residues, Lys^{24}, His^{25}, and Lys^{27}, were substituted with a Ser residue in SDF-1 (3/6), leading to the previously reported impaired interaction of this mutated chemokine with heparin (Amara et al. 1999; Sadir et al. 2001). It is clear from these results that these three basic residues were essential for the promotion of dimerization of SDF-1 by oligosaccharides. In addition to the mutated amino acids, other basic residues, Lys^{1}, Arg^{41}, and Lys^{43}, have also been shown to contribute to the interaction with heparin. However, the absence of complex detected by MS suggested that the interactions involving these residues in the modified chemokine were not stable enough to be preserved in the electrospray source.

It has been previously demonstrated that SDF-1 induced the migration and the invasion of human hepatoma Huh7 cells in a dose-dependent manner (Sutton et al. 2007). We have carried out the same migration and invasion assays with SDF-1 (3/6). The Huh7 cell migration across fibronectin and the invasion through a reconstituted extracellular matrix induced by SDF-1 (3/6) were decreased by 50 ± 9% and 46.7 ± 10.1%, respectively (Figure 5) as compared to those induced by the wild-type chemokine ($P < 0.01$, $n = 3$). This decreased chemotactic activity and the loss of heparin binding capacity of SDF-1 (3/6) suggested that glycosaminoglycan–chemokine interactions are required to induce an optimal chemotactic response.

In order to address the specificity of the oligosaccharide binding and its role in the chemokine dimerization, we have analyzed by ACE/MS, the interaction of SDF-1 with a synthetic sulfated fucose pentasaccharide resembling the repeating unit of algal fucoidan (Scheme 1A). The many biological activities exhibited by fucoidan toward mammalian systems resulting from its interaction with a variety of proteins may offer therapeutic alternatives for fucoidan as a glycosaminoglycan mimetic. An ACE/MS experiment with 3 μM of fucoidan pentasaccharide (MW = 1420.12 g mol^{-1}) in the electrolyte led to a mass spectrum exhibiting three charge state distributions (Figure 6). In addition to the monomeric SDF-1 (charge state distribution from +5 at *m/z* 1593.9 to +3 at *m/z* 2651.4, corresponding to

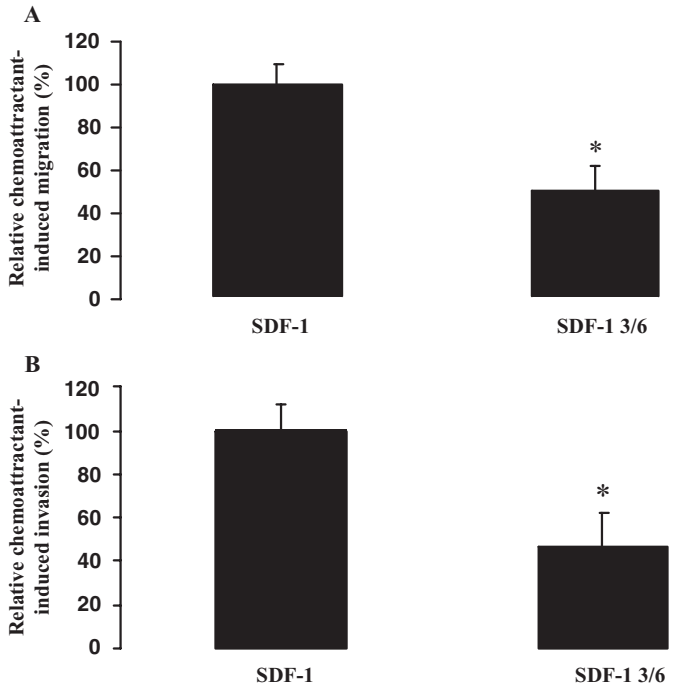


Fig. 5. Compared in vitro chemotactic activities of SDF-1 and SDF-1 (3/6). Induction by the chemokines of (**A**) the migration of Huh7 cells across fibronectin and (**B**) the invasion of Huh7 cells through a reconstituted extracellular matrix. Cell migration or invasion induced by SDF-1 was set to 100%. *, $P < 0.05$.

a calculated experimental mass of 7958.3 ± 6.7 g mol^{-1}), two other species were observed with respective ions of charge states +5 (*m/z* 1876.8) and +4 (*m/z* 2344.8) corresponding to a molecular mass of 9377.1 ± 2.7 g mol^{-1} and four ions with a charge state distribution from +9 (*m/z* 1926.4) to +6 (*m/z* 2887.6) corresponding to a molecular mass of 17327.3 ± 7.8 g mol^{-1}. The molecular mass of 9377.1 g mol^{-1} corresponded to a 1/1 SDF-1/fucoidan pentasaccharide complex, while the molecular mass of 17327.3 g mol^{-1} corresponded to a 2/1 SDF-1/fucoidan pentasaccharide complex. Compared to heparin, fucoidan led to a more heterogeneous population of noncovalent complexes. Fucoidan was still able to promote dimerization of chemokine, but the detection of the 1/1 complex suggested that this dimer is less stabilized by fucoidan, likely due to the less specific interaction.

Structure of the bound oligosaccharide in the complex with a SDF-1 dimer

In order to delineate the structural features of bound oligosaccharides, especially as regards their sulfation level, we performed detailed analysis of the major charge state for each detected 2/1 complex. The major ion for the complex between the dimer of SDF-1 and the tetrasaccharide was of the charge state +8 (Figure 3B). Actually, the expanded spectrum around this charge state revealed three groups of ions, A, B, and C, which differed by 10 *m/z* units as observed for the major ions at *m/z* 2123.5, 2133.2, and 2143.7, respectively (Figure 7A). Given the charge state +8, this interval corresponded to 80 mass units, that is the mass of a sulfate group SO_3. Hence, the ion group C was the most sulfated, carrying one more sulfate group than ions B and two more sulfate groups than ions A.

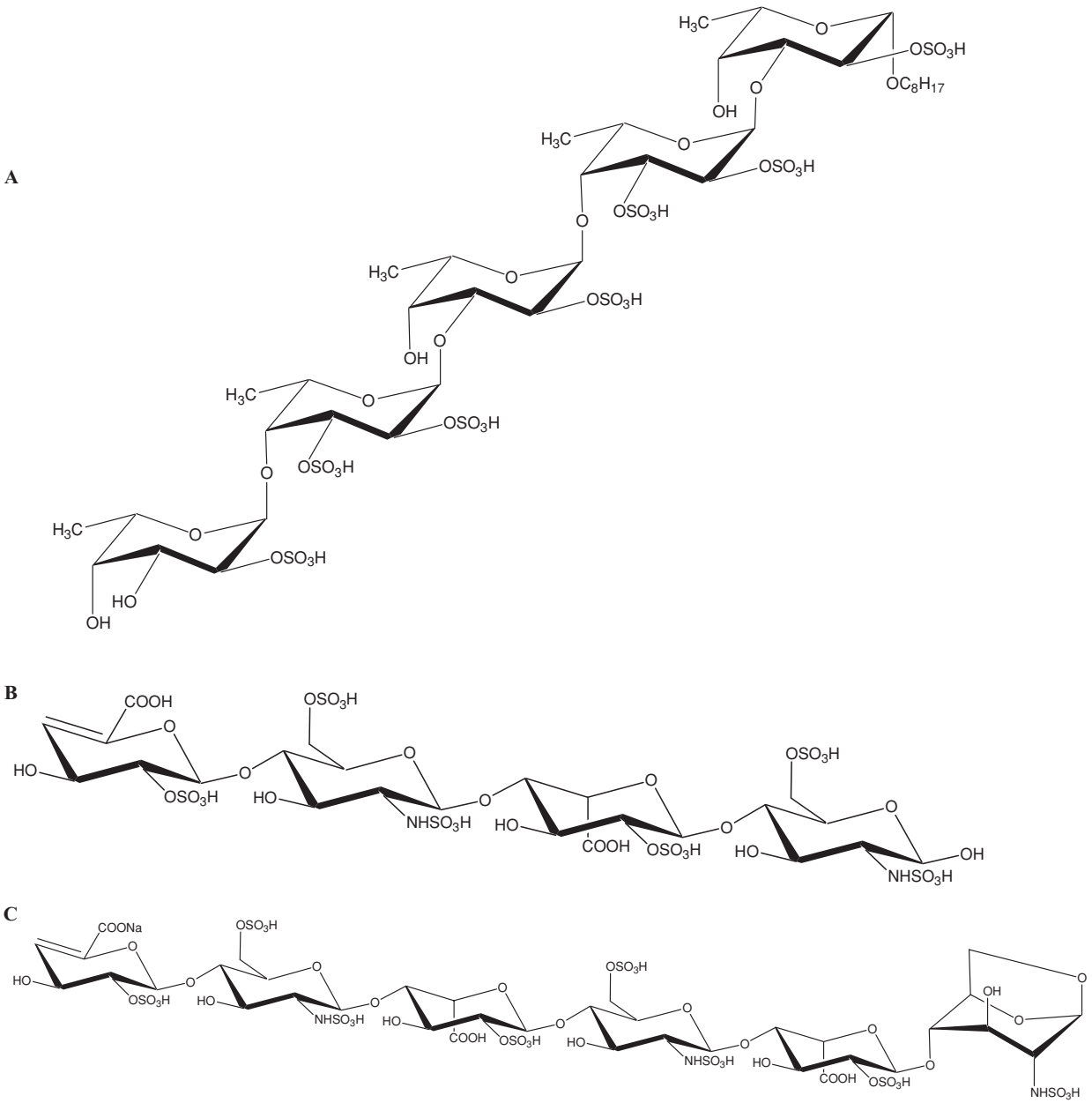
Inside each group, B and C, different ions were distinguished that were attributed to sodium adducts (Figure 7). For group B, the major ion was at *m/z* 2133.2 yielding a calculated molecular mass of 17,057.6 g mol^{-1}. Given the experimental mass of SDF-1 (7953.4 ± 5.0 g mol^{-1}), a mass value of 1150.8 g mol^{-1} was deduced for the bound tetrasaccharide. This molecular mass could correspond to a tetrasaccharide with six sulfate groups (theoretical average molecular mass of 1154.9 g mol^{-1}), a possible structure of which is shown in Scheme 1B. Similarly, the major ions at *m/z* 2123.5 and 2143.3 of the groups A and C corresponded to a bound tetrasaccharide with five and seven sulfate groups, respectively. The last one may represent a rare sulfation pattern including a 3-*O*-sulfate that is present in enoxaparin and that could be selected by SDF-1 upon interaction with the starting oligosaccharides.

As regard to interaction with heparin hexasaccharide, the major ion for the complex between the dimer of SDF-1 and this oligosaccharide was also of the charge state +8 (Figure 3C). The expanded spectrum around this charge state revealed three groups of ions, D, E, and F, which differed by 10 *m/z* units, as observed for their major ions at *m/z* 2185.9, 2195.6, and 2205.4, respectively (Figure 7B). As for the tetrasaccharide, these ions were then different in their sulfate content. These major ions were attributed to sodium adducts bearing one sodium atom as counter-ion on the hexasaccharide. The major ion at *m/z* 2195.6 of the group E yielded a calculated molecular mass of 17556.8 g mol^{-1} for the 2/1 SDF-1/hexasaccharide complex, from which a molecular mass of 1649.2 g mol^{-1} was deduced for the bound hexasaccharide, with respect to the experimental mass of SDF-1 (7953.8 ± 5.2 g mol^{-1}). This mass value could corresponded to a hexasaccharide with eight sulfate groups and one sodium counter-ion (theoretical average molecular mass of 1656.3 g mol^{-1}), a possible structure of which is shown in Scheme 1C. The tetra- and hexasaccharide fractions used in this study were derived from the low molecular weight heparin enoxaparin obtained by alkaline depolymerization of heparin, a method leading to unsaturation at the nonreducing end of oligosaccharides. In addition, 15–25% of the chains exhibit 1,6-anhydro bridge at the reducing end (Mascellani et al. 2007), as shown for the hexasaccharide in Scheme 1C. The major ions at *m/z* 2185.9 and 2205.4 of the other groups D and F were attributed to a monosodiated hexasaccharide with seven and nine sulfate groups, respectively.

Derived from depolymerized heparin, the size-homogeneous tetra- and hexasaccharide fractions exhibited the heterogeneous sulfate content. In addition to the detection of protein–carbohydrate complexes and determination of their stoichiometry, the described ACE/MS analysis also allowed resolving the sulfate heterogeneity of the saccharide ligands.

Discussion

The dimerization of chemokines like SDF-1 upon interaction with glycosaminoglycans is currently a well-accepted hypothesis, despite the fact that no evidence has been provided to date of such dimer formation in a physiological setting. Consequently, the functionally active form of SDF-1 is controversial, the previous studies describing either a monomer or a dimer, or equilibrium between both forms. The results reported in this study provide the first evidence that GAGs binding and



Scheme 1. Structural representation of the synthetic fucoidan pentasaccharide (A), and of the proposed SDF-1-bound heparin tetrasaccharide (B) and hexasaccharide (C) from mass spectra Fig. 7A and Fig. 7B, respectively.

oligomerization are coupled at physiological concentrations and that a dimer exists in an oligosaccharide bound form. Unlike many other chemokines such as MIP-1β or MCP-1, which easily dimerize given the low Kd values of their dimer (0.1–10 μM) (Chakravarty et al. 1998; Laurence et al. 2000; Yu et al. 2005), SDF-1 appears to be mostly monomeric in solution with respect to the mM range of the dimer Kd value. As a matter of fact, dimer has been observed in NMR experiments only with SDF-1 in mM concentration (Baryshnikova and Sykes 2006). However, it has been shown that the Kd value can be lowered to about 150 μM in slightly acidic pH and in the presence of sulfate anion in the buffer (Veldkamp et al. 2005). Therefore, we have studied the effect of sulfated oligosaccharides on SDF-1 and their ability to promote dimerization. For that purpose, we have used a new analytical procedure based on the hyphenation between CE and MS. CE allowed analyzing SDF-1 in μM range concentrations, i.e. lower values than the mM range values reported before in NMR, light scattering, and ultracentrifugation experiments (Holmes et al. 2001; Gozansky et al. 2005; Baryshnikova and Sykes 2006; Murphy et al. 2007). Furthermore, CE was used in the affinity mode, which enables biomolecular interactions without prior immobilization of the interacting molecules. Coupled to MS, this method allowed the study for the first time of the effect of size-defined oligosaccharides on the quaternary organization of SDF-1.

In the absence of sulfated oligosaccharides, SDF-1 at the concentration of 15 μM was detected as a monomeric form. This result was expected given this concentration of SDF-1 that was

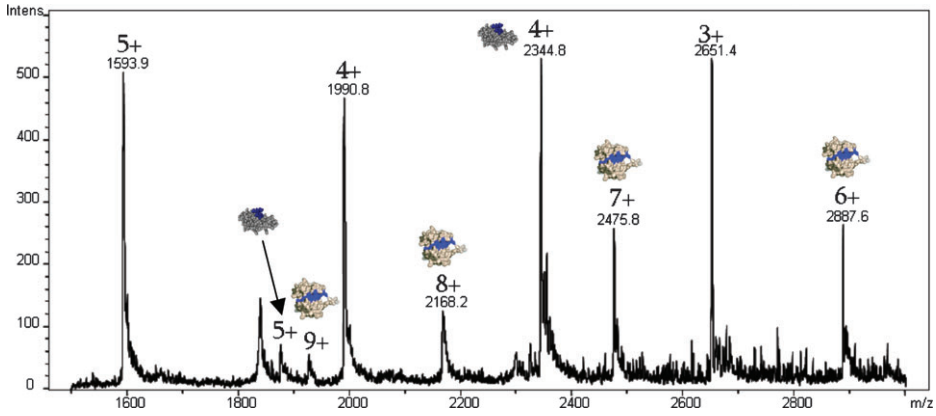
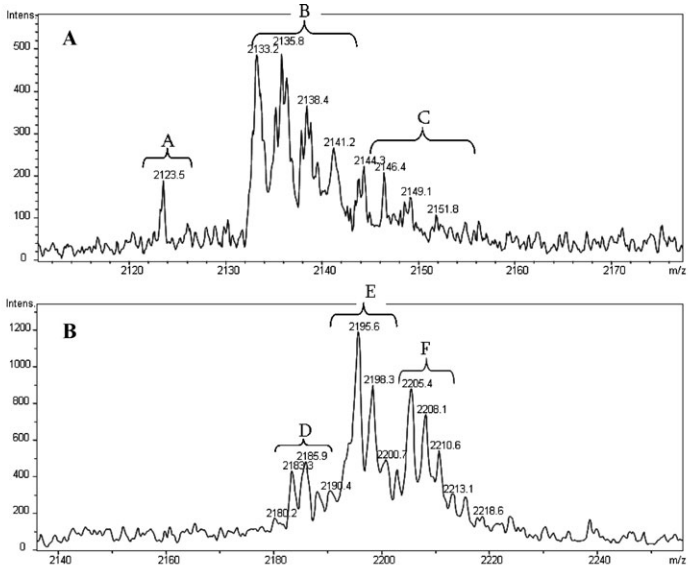


Fig. 6. Positive ESI mass spectrum of SDF-1 upon interaction with fucoidan pentasaccharide acquired by affinity capillary electrophoresis-mass spectrometry (ACE-MS). Affinity capillary electrophoresis of SDF-1 was carried out in the presence of 3 μM fucoidan pentasaccharide in the electrolyte. SDF-1 monomer and dimer structures represented above the corresponding ions are from (Handel et al. 2005). CE conditions: same as in Figure 3.



m/z (ions group)	Complex mol. weight	Mol. weight	Bound oligosaccharide Sulfate content	Na adducts
2133.2 (B)	**17057.6**	**1150.8**	**Tetrasaccharide (Scheme 1B)**	**6 SO_3**
2135.8 (B)	17078.3	1171.5	6 SO_3	+ Na - H
2138.4 (B)	17099.1	1192.3	6 SO_3	+ 2Na - 2H
2141.2 (B)	17121.5	1214.7	6 SO_3	+ 3Na - 3H
2123.5 (A)	16979.9	1073.1	5 SO_3	
2143.7 (C)	17140.7	1233.9	7 SO_3	
2146.4 (C)	17163.1	1256.3	7 SO_3	+ Na - H
2148.8 (C)	17182.3	1275.5	7 SO_3	+ 2Na - 2H
2195.6 (E)	**17556.8**	**1649.2**	**Hexasaccharide (Scheme 1C)**	**8 SO_3 + Na – H**
2198.3 (E)	17578.3	1670.7	8 SO_3	+ 2Na - 2H
2200.7 (E)	17597.5	1689.9	8 SO_3	+ 3Na - 3H
2183.3 (D)	17458.3	1550.7	7 SO_3	
2185.9 (D)	17479.1	1571.5	7 SO_3	+ Na - H
2205.4 (F)	17635.1	1727.5	9 SO_3	+ Na - H
2208.1 (F)	17656.7	1749.1	9 SO_3	+ 2Na - 2H
2210.6 (F)	17676.7	1769.1	9 SO_3	+ 3Na - 3H

Fig. 7. Focus of the ACE-MS spectra on the major ions of the charge state +8 for the 2/1 complexes between SDF-1 and heparin oligosaccharide. (**A**) Heparin tetrasaccharide and (**B**) Heparin hexasaccharide. The molecular weights of the corresponding noncovalent complexes and of bound oligosaccharide are indicated in the table underneath. The sulfate content and Na adducts of the bound oligosaccharides are indicated in the right column. Data in boldface correspond to the structures exhibited in Scheme 1 for the sulfated tetrasaccharide and hexasaccharide.

ten times lower than the lowest published Kd values; in addition, the neutral pH of the separation electrolyte did not favor the dimer formation (Veldkamp et al. 2005). Therefore, SDF-1 is clearly monomeric in solution in μM concentration. The presence of heparin disaccharide I-S leads to the detection of a 1/1 complex between SDF-1 and the oligosaccharide, indicating that this noncovalent complex is stable enough to be transferred from solution to gas phase. While an octasaccharide was previously determined as the minimal length for the binding to SDF-1 (Sadir et al. 2001), this result shows that an oligosaccharide as small as a sulfated disaccharide is able to bind to the chemokine. Surprisingly, no such 1/1 complex was observed with heparin tetra- and hexasaccharide. A SDF-1/oligosaccharide 2/1 complex was instead detected, indicating that the binding of these oligosaccharides promoted the dimerization of SDF-1. It is worthy to note that the free SDF-1 dimer was not detected with these longer oligosaccharides, confirming that the monomeric form is the metastable state of SDF-1 and that the dimer requires to be stabilized by a long enough bound oligosaccharide.

An oligosaccharide as small as a tetrasaccharide was able to stabilize the SDF-1 dimer. This stabilizing effect seemed to be enhanced by hexasaccharide based on the intensities of the corresponding ions, suggesting a dependence on oligosaccharide length. It has been proposed from previous structural models that dodeca- to tetradecasaccharides have an optimal length to span along the whole dimer interface (Sadir et al. 2001). The fact that the monomeric form of SDF-1 was still detected in the MS spectra with hexasaccharide may indicate either dimer dissociation in the electrospray source, or a partial dimerization upon interaction with this oligosaccharide during ACE. Further investigations are currently in progress to determine the effect of longer oligosaccharides.

The stoichiometry of the observed 2/1 SDF-1/sulfated oligosaccharide complexes are in agreement with the structural model previously proposed, in which the dimer interface constitutes a crevice bordered by the basic residues $Lys^{24}/Arg^{41}/Lys^{27}/Lys^{43}$ and is thus suitable for the binding of one sulfated oligosaccharide chain (Sadir et al. 2001; Lortat-Jacob et al. 2002). It is likely that these residues on each site of the protein–protein interface establish salt bridges with sulfate groups of the oligosaccharide, requiring the appropriate orientation of the sulfate group toward both sites of the interface to lead to the stabilization of the dimer. How the sulfate pattern governs the interaction between HS and protein in a specific way is not well understood and is currently a matter of some controversy, especially with respect to the fibroblast growth factors (Kreuger et al. 2001; Mulloy 2005; Imberty et al. 2007). The synthetic fucoidan pentasaccharide has about the same degree of sulfation as the heparin oligosaccharides. However, unlike heparin oligosaccharide, the fucoidan pentasaccharide induced the formation of both 1/1 and 2/1 SDF-1/sulfated oligosaccharide complexes. Hence, fucoidan interacts in a less specific manner, pointing out the importance of the regio- and stereo-selective orientation of the sulfate groups to drive the dimer formation.

We did not observe the interaction between SDF-1 (3/6) and hexasaccharide, confirming the role of the basic residues Lys^{24} and Lys^{27} of the first β-strand in the binding of carbohydrates. Besides, no dimer was detected with this mutated form of SDF-1. It is likely that in the absence of bound oligosaccharide, the formation of dimer cannot be driven by the stabilizing effect of the sulfated ligand.

In agreement with the recent in vitro and in vivo studies, our data further support the important role of GAG/chemokine interactions in modulating the SDF-1-mediated biological activities (Santiago et al. 2006; Mellor et al. 2007; Murphy et al. 2007; Sutton et al. 2007). The mutated form of SDF-1, SDF-1 (3/6), exhibited a 50% lower chemotactic activity compared to the wild-type chemokine. On the other hand, it has been previously demonstrated that the affinity of SDF-1 (3/6) for CXCR4 was not affected (Amara et al. 1999). It is possible that the impaired dimerization of SDF-1 (3/6) could be one of the mechanisms which underlies its lower chemotactic activity. It has been previously shown that SDF-1 triggers CXCR4 receptor dimerization, an important process for the signaling pathway (Vila-Coro et al. 1999). Therefore, cell surface GAGs may modulate in vivo the function of the chemokine by promoting and presenting the dimeric form to the receptor, and then amplifying the signal transduction. SDF-1 is constitutively expressed, whereas the cell surface HS exhibits sulfate patterns and organization in discrete sulfated and unsulfated domains that are specific to the cell type, to the developmental status and to the normal or pathological state of the cell (Gama et al. 2005). Therefore, cell surface HS may contain informational code in its sequence and domain organization allowing the cell to control the action of diverse signaling molecules like chemokines, through localized gradient of bound chemokine, induced-conformational change, dimerization and functional protein domain formation, and selective receptor binding. This HS code remains to be deciphered to orientate the design carbohydrate-based bioactive compounds targeting the GAG–chemokine interaction.

Material and methods

Reagents

Recombinant SDF-1 (SDF-1α, residues 1–68) was purchased from PeproTech (France) and diluted to 75 μM in water (MW 7959.4 g mol^{-1}). The mutant SDF-1 (3/6) (25 μM in water), in which a cluster of basic residues Lys^{24}, His^{25}, and Lys^{27} were substituted with a Ser residue and Lys^{68} was suppressed (MW 7699.0 g mol^{-1}), has been synthesized as previously described (Amara et al. 1999). Heparin disaccharide I-S was purchased from Sigma-Aldrich (Lyon, France) (MW 577 g mol^{-1}). Heparin tetra- and hexasaccharides were prepared from Enoxaparin by preparative gel permeation chromatography as previously described (Mulloy et al. 1997). The synthetic fucoidan pentasaccharide (MW 1420.12 g mol^{-1}) was synthesized as previously reported (Hua et al. 2004). Polyethylene oxide (PEO, M_r 200,000) was from Sigma-Aldrich. Other chemicals and reagents were obtained from commercial sources at the highest purity available. All buffers and solutions were prepared using ultra-pure water (Milli-Q, Millipore, Milford, MA) and degassed by filtration through 0.2 μm filter units before use.

CE-MS experiment

Capillary electrophoresis $HP^{3D}CE$ (Agilent Technologies, Waldbronn, Germany) device was coupled to an Esquire 3000+ ESI source ion trap mass spectrometer (Bruker Daltonics, Wissembourg, France) using capillaries of 98 cm total length (21.5 cm to the UV detector) connected to the mass spectrometer through a coaxial sheath-flow interface G1607A (Agilent Technologies). Agilent ChemStation software was used for the

CE-MS system control, data acquisition, and data analysis. Bare fused-silica capillaries (Phymep, Paris, France) of 50 μm i.d. (360 μm o.d.) were used. The capillaries were conditioned by successive 15 min flushes (1 bar) with water, 1 M NaOH, 1 M HCl, and water. Dynamic coating of the capillary with PEO was carried out prior to each sample injection as previously described (Fermas et al. 2008). After each separation, the capillary was washed with water, 0.1 M NaOH, and water for 10 min per step. Once the separation was finished, the capillary was washed by injections of water/NaOH/water, and then capillary was ready for another PEO coating. The separation electrolyte was a 75 mM ammonium acetate buffer, pH 6.5, containing sulfated oligosaccharide at various concentrations (1×10^{-6} to 15×10^{-6} M). Samples were introduced in the hydrodynamic mode. The injection protocol was composed of four successive hydrodynamic injections: the neutral marker benzyl alcohol (50 mbar for 2 s), the separation electrolyte (30 mbar for 2 s), the sample (50 mbar for 30 or 60 s), and the separation electrolyte (30 mbar for 2 s). Separations were performed under a positive voltage of 10 kV and a pressure of 50 mbar. The temperature of the samples and the capillary cartridge were set at 25°C. The capillary effluent was mixed with 10% methanol in water using a syringe pump (74,900 series, Cole-Palmer) at a flow rate of 3 μL min^{-1}. The drying gas (nitrogen) temperature was maintained at 100°C, with a flow rate of 3 L min^{-1}. The nebulizing gas (nitrogen) pressure was optimized at 5 psi. The instrument was calibrated using standards ES tuning mix (Agilent Technologies) as external mass standards. The scan mass spectra were obtained in the standard mode (50–3000 *m*/*z*). Data acquisition was performed in the standard resolution mode with a scan speed of 13,000 *m*/*z* per second. The ESI mass spectra were obtained in a positive ionization mode. The target mass was optimized at 3000 *m*/*z*. Other parameters were optimized in order to get the best signal-to-noise ratio.

Cell migration and invasion assays

Human hepatoma Huh7 cells were grown as described (12). Cell migration or invasion was performed using Bio-coat cell migration chambers (Becton Dickinson, Pont-de-Claix, France), as described (Sutton et al. 2007). Briefly, inserts were coated with fibronectin (100 μg/mL, Santa Cruz Biotechnology) for migration or Matrigel (320 μg/mL, BD Bioscience Pharmingen) for invasion assay. SDF-1/CXCL12 or its modified form SDF-1 (3/6) was added to the lower chamber. After 24 h, cells that had migrated through the filter pores were fixed with methanol, stained with Mayer's hemalum, and counted. For the determination of statistical significance, an ANOVA test was performed with the Statview software. A P value of <0.05 was used as the criterion of statistical significance.

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Conflict of interest statement

None declared.

Abbreviations

ACE, affinity capillary electrophoresis; CE, capillary electrophoresis; ESI, electrospray ionization; GAG, glycosaminoglycan; HS, heparan sulfate; MS, mass spectrometry; PEO, polyethylene oxide; SDF, stromal cell-derived factor.

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