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BRIEF REPORT

Structural requirements of glycosaminoglycans for their interaction with HIV-1 envelope glycoprotein gp120

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Abstract Heparan sulfate proteoglycans are known to assist HIV-1 entry into host cells, mediated by the viral envelope glycoprotein gp120. We aimed to determine the general structural features of glycosaminoglycans that enable their binding to gp120, by surface plasmon resonance. Binding was found to be dependent on sequence type, size and sulfation patterns. HIV-1 gp120 prefers heparin and heparan sulfate (with at least 16 monomers in length) over chondroitin and dermatan. Sulfate groups were essential to promote this interaction. These results advance the understanding of the molecular-level requirements for virus attachment and cell entry.

Keywords HIV-1 · gp120 · Viral attachment · Heparan sulfate proteoglycans · Glycosaminoglycans · Surface plasmon resonance

Introduction

Human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (gp120 and gp41) are the prime mediators of the capability of the virus to bind and enter into host $CD4^+$ cells. These viral glycoproteins are required for engagement with the target cell receptors CD4 and co-receptors CCR5 or CXCR4 and, since HIV-1 is an enveloped virus, to drive the fusion between the viral and cell membranes [1, 2]. However, additional mediators at the cell surface help the virus to approach and attach to the target cells before CD4 interaction [3]. These attachment mediators include adhesion molecules, lectins, heparan sulfate proteoglycans (HSPGs), and the glycolipid GalCer (galactosylceramide). HSPGs are proteins (either membrane-bound or secreted) with attached glycosaminoglycan (GAG) chains [4]. These GAGs are linear polysaccharides consisting of repeating disaccharide units of hexosamine and uronic acid, which are often sulfated and/or acetylated. Syndecans are a transmembrane form of HSPG that have an extended ectodomain, with the heparan sulfate (HS) chains mainly attached at a distal position from the membrane [4]. HS is the major GAG of cell-surface proteoglycans. Chondroitin and dermatan sulfate are mainly associated with the proteoglycans from the extracellular matrix and are important constituents of blood vessels, heart valves, bone, cartilage, tendons and skin [5].

In vitro studies of glycosaminoglycan-protein interactions usually rely on soluble heparin as a GAG model. Heparin is a highly sulfated form of heparan that is produced by mast cells to act as adjuvant in anti-coagulation [5]. Heparin and HS have been shown to bind HIV-1 gp120 [6, 7], and the essential residues for this interaction have been identified, included in the V2 and V3 loops, in the C-terminal domain, and in the CD4-induced co-receptor binding site [8–10]. Cells treated with heparitinase [11] or knocked out for GAG production [12] have much lower levels of viral attachment and infection than their wild-type counterparts. Syndecans have also been implicated in

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attachment and trans-infection to T lymphocytes [13]. Moreover, syndecans present in macrophages are crucial for HIV-1 attachment, as they compensate for the lower CD4 levels of these cells compared to $CD4^+$ T cells [14]. Syndecan-3 was also identified as a specific attachment site during HIV-1 infection of dendritic cells [15]. The ubiquitously present proteoglycans, especially in epithelial, endothelial cells and dendritic cells, can efficiently capture HIV virions, protect them, and facilitate trans-infections to T lymphocytes, enhancing viral spreading and pathogenesis [16].

In this work, we explored the interaction of HIV-1 gp120 with distinct glycosaminoglycans that differed in their type/composition, sulfation pattern and size. We screened these molecules for gp120 binding via surface plasmon resonance (SPR), a technique that allows label-free characterization of molecular interactions, in order to understand the general structural requirements for glycosaminoglycan-HIV-1 envelope binding that may contribute to the design of more efficient blockers of HIV binding.

Materials and methods

High-grade heparin from porcine mucosa, heparin oligosaccharides (dp4, dp8, dp12, dp16, dp20 and dp30, with dp stating the monomer number), selectively desulfated heparins (2-*O*-desulfated, 6-*O*-desulfated, *N*-desulfated and *N*-desulfated re-*N*-acetylated), heparan and dermatan sulfate from porcine mucosa, and K5 polysaccharide were all from Iduron (Manchester, UK). Chondroitin sulfate from shark cartilage and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA).

Recombinant HIV-1 BaL gp120 envelope glycoprotein (produced in HEK-293), in monomeric form, was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH; Germantown, MD, USA).

Biacore CM5 sensorchips, the amine coupling kit (containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride [EDC] and N-hydroxysuccinimide [NHS], in 1 M ethanolamine-HCl, pH 8.5), and HBS-EP running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005 % v/v surfactant P20) were from Biacore GE Healthcare Life Sciences (Uppsala, Sweden). All experiments were performed using a Biacore 3000 SPR instrument (GE Healthcare).

BSA or recombinant HIV-1 gp120 were immobilized on CM5 sensorchips via amine coupling. The chip surface was activated with a freshly prepared 1:1 mixture of EDC and NHS (50 μL), followed by injection of 50 μg/mL of protein in acetate buffer, pH 4.8 (60 μL) and blocking of the remaining active surface with 1 M ethanolamine-HCl, pH 8.5 (60 μL). The flow was kept at 5 μL/min during the immobilization procedures.

To assess the influence the positive-charge distribution of gp120, we employed two other immobilization methods that required pre-incubation of the proteins with *p*-nitrocatechol sulfate (pNCS, Sigma) or suramin (Tocris Bioscience, Bristol, UK) at room temperature. The pre-incubation of gp120 with pNCs was done at 50 μg/ml protein concentration with a protein:pNCS molar ratio of 1:10 for 25 min, followed by immobilization with acetate buffer, pH 4.0. For suramin, the concentration of protein was doubled to 100 μg/mL, and the molar ratio for incubation was 1:5 (protein:suramin), for 1 h, with subsequent immobilization done at pH 4.0. Binding experiments were performed in the running buffer HBS-EP.

Results

Measuring the binding of proteins to glycosaminoglycans in SPR systems usually relies on the immobilization of biotinylated GAG (usually heparin) at the sensor surface via streptavidin linking and flowing the protein as the analyte. This is because the heterogeneity of molecular masses and the optical properties of GAG in solution may not allow changes to be detected adequately by the SPR sensor. However, since our objective was to test a diverse panel of different GAGs under equivalent conditions, it was more appropriate to use them as analytes against the HIV-1 envelope glycoprotein. This strategy has the analytical disadvantage of lower-quality sensorgrams (in comparison to the reverse situation), which are required for detailed kinetic studies using SPR. Hence, in this work, we focused on a qualitative comparison of the binding of each of the tested GAGs.

In order to establish the requirements for GAG binding to HIV-1 gp120, we selected a panel of soluble GAGs differing in their type (monomer sequence), desulfation pattern and oligomer size. We initially selected four GAGs, namely heparin, heparan, chondroitin and dermatan sulfate, which differ in their disaccharide unit sequence, as well as in their distinct functions and locations in the human body. To assess the importance of the sulfate groups in the backbone of the GAG, we used heparins that were selectively desulfated at the position 2-*O* or 6-*O* and an *N*-desulfated, re-*N*-acetylated GAG. As a completely non-sulfated control, we used the K5 polysaccharide from *Escherichia coli*, a nonsulfated analogue of HS. Furthermore, we also tested different sizes of heparin oligosaccharides, ranging from dp4 to dp30.

Immobilization of HIV-1 gp120 BaL via amine coupling on carboxymethylated dextran surfaces was readily achieved, with immobilization levels around 1.6×10^4 RU. Heparin, which is known to bind gp120 [6], was

consistently the best binder to gp120 among the soluble GAGs tested. Of the different types of GAG (Fig. 1A, D), HS followed heparin in affinity, while dermatan and chondroitin had lower binding. Comparing heparin with its selectively desulfated counterparts and the nonsulfated K5 (Fig. 1B, E), it can be observed that all of the desulfated forms have considerably lower (almost residual) binding to gp120. However, the distinction between them is not clear. The binding of heparin is also size-dependent (Fig. 1C, F), with a higher molecular mass associated with a higher degree of binding in the association phase. The binding was weak below dp16, indicating that 16 monomers is the minimum needed for significant binding.

The direct immobilization of gp120 on the CM5 chip is directed by the electrostatic attraction to the negatively charged carboxymethylated surface (at pH > 3) before the covalent reaction occurs. To determine whether any positively charged patches on the surface of gp120 were hidden following the immobilization procedure, we undertook a protection strategy by incubating with sulfated compounds prior to the immobilization. For this, we used the monosulfated small molecule *p*-nitrocatechol sulfate and the intermediate-sized hexasulfated suramin. The immobilization levels obtained when gp120 was pre-incubated with the sulfated compounds was around 1.1×10^4 RU. A significant reduction was observed when compared with the levels of the unprotected compounds (1.6×10^4 RU), indicating that these compounds decrease the exposure of the glycoprotein-positive patches, hence the attraction to the surface and thus the immobilization efficiency.

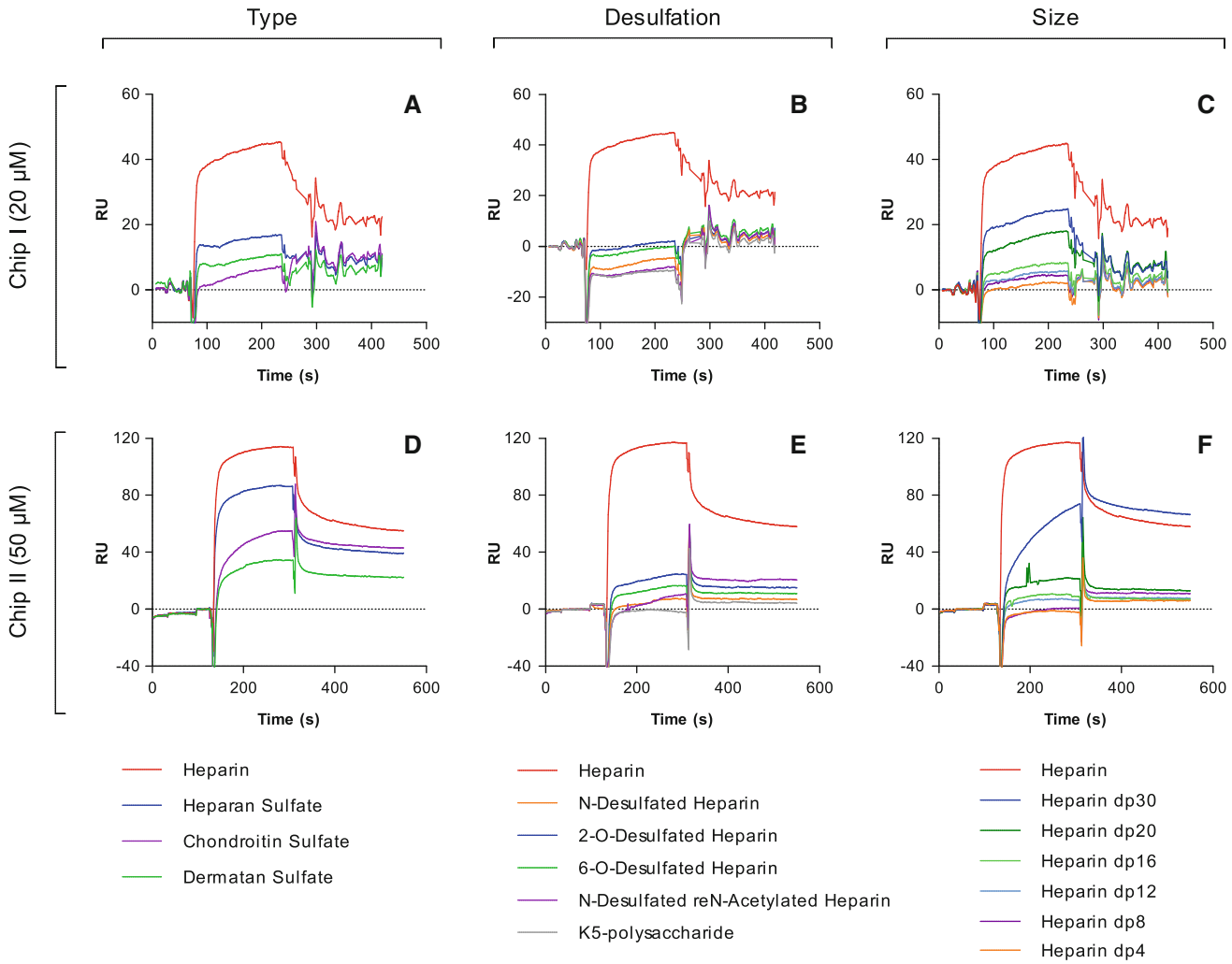

Fig. 1 Sensorgrams of glycosaminoglycans binding to immobilized HIV-1 gp120 BaL. All of the soluble GAGs were divided in three categories and tested against two different chips with different protein lots (chip I and II). Immobilized BSA was used as a control surface, and all of the traces had this control subtracted. (**A**–**C**) For chip I, GAGs were used at 20 µM, with a flow rate of 10 µL/min; immobilization levels were 19,835 RU for BSA and 16,103 RU for gp120. (**D**–**F**) For chip II, GAG were used at 50 µM, with a flow rate of 40 µL/min; immobilization levels were 15,094 RU for BSA and 16,482 RU for gp120. Plots are representative of duplicate experiments

However, the GAG interactions in the protected immobilized gp120 did not show significant differences (Fig. 2). Heparin was again the GAG type that bound with highest affinity, followed by heparan (based only on the association rate). Dermatan and chondroitin affinities seem also to depend on the type of protection applied. Desulfated heparins (Fig. 2B and E) bound to a much lower degree than heparin itself, as also shown in Fig. 1. In the case of suramin-treated gp120 (Fig. 2E), the binding of desulfated GAG was at residual levels. However, for pNCS-treated gp120 (Fig. 2B), the levels of binding of these compounds in the association phase are higher when compared to suramin, with 2-*O*-desulfated heparin having the highest affinity among them and K5 the lowest. Nevertheless, in the dissociation phase, the levels were still low when compared, for example, with the other GAGs shown in Fig. 2A. Size-dependent binding was also retained after protection, with the binding threshold maintained at dp16 (Fig. 2C, F).

Discussion

In this study, we sought to rank different glycosaminoglycans qualitatively for their binding to HIV-1 gp120, under identical circumstances, using surface plasmon resonance. Despite the analytical challenge of directly monitoring the binding of compounds with little direct impact on the dielectric constants of the solutes, by running different GAGs on immobilized gp120, we observed that the binding was dependent on GAG type (sequence), desulfation pattern, and size. Heparin was the best binder for

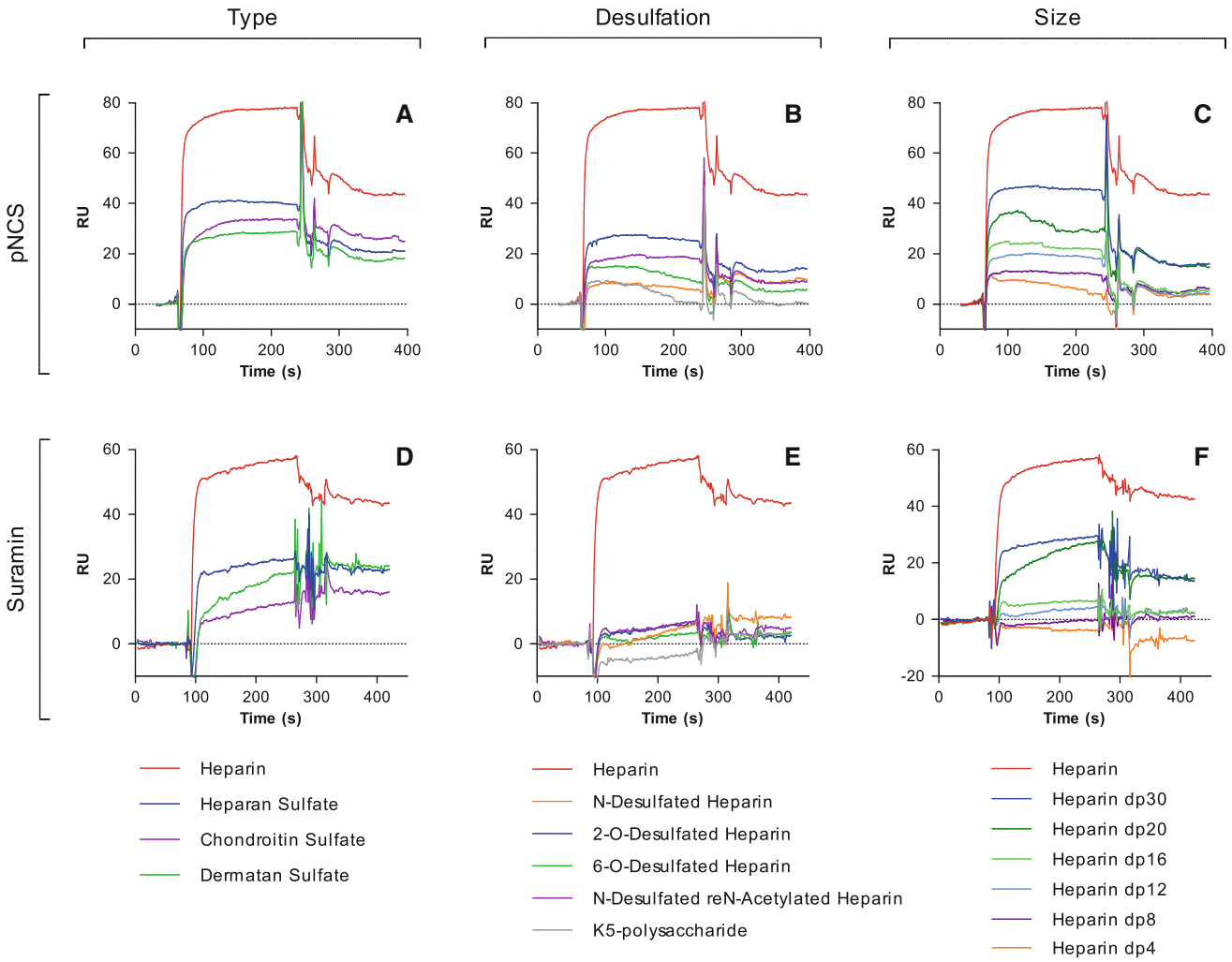

Fig. 2 Sensorgrams of glycosaminoglycans binding to HIV-1 gp120 BaL pre-protected with sulfated compounds upon immobilization. The protein was incubated with *p*-nitrochatechol sulfate (**A**–**C**) or suramin (**D**–**F**), as described in the text, before being injected for immobilization. GAGs were used at 20 μM, with a flow rate of 20 μL/min. Traces are subtracted for the control surface (BSA for suramin chip and activated/deactivated surface for pNCS chip). Immobilization levels for the pNCS chip were 11,400 RU for gp120, and for the suramin chip they were 11,305 RU for gp120 and 9,970 RU for BSA. Plots are representative of duplicate experiments

Table 1 Molecular weights of the glycosaminoglycans and their interaction with gp120

	Glycosaminoglycan	Mean M_w[a]	Binding to gp120
Sequence-distinct GAG	Heparin	15,000	+++
	Heparan sulfate	22,000	++
	Dermatan sulfate	41,400	+
	Chondroitin sulfate	30,000	+
Heparin oligomers	Heparin dp4	1340	–
	Heparin dp8	2680	–
	Heparin dp12	4020	+
	Heparin dp16	5360	+
	Heparin dp20	6700	++
	Heparin dp30	>9000	++
Desulfated GAG	2-*O*-desulfated heparin	15,000	–
	6-*O*-desulfated heparin	15,000	–
	N-desulfated heparin	15,000	–
	N-desulfated re-*N*-acetylated heparin	15000	–
	K5 polysaccharide	30000	–

[a] Some values are estimates provided by the supplier

gp120 (Table 1). It was also the most highly sulfated GAG among the tested panel and the best-characterized GAG binding partner of gp120 [6, 7]. Regarding the types of GAG, HS followed heparin in affinity, with dermatan and chondroitin possessing lower binding ability. The distinction between dermatan and chondroitin also depended on the protection of gp120 upon immobilization, but chondroitin usually showed higher RU levels in the dissociation phase. We can rank the binding as heparin > heparan > chondroitin ≈ dermatan. This is consistent with studies showing the ability of syndecans to capture HIV, since HS is the main GAG in those proteoglycans [13]. Chondroitin and dermatan sulfates are less relevant in the context of HIV infection, as their locations and functions tend to be more at the level of the extracellular matrix [5].

Desulfated heparins showed residual levels of binding, with little distinction observed among them. The completely unsulfated K5 had the lowest absolute binding level. This confirms the crucial role of the GAG sulfate groups for their proper function and binding abilities, especially towards our protein of study, gp120. Desulfated heparins, especially *O*-desulfated ones, were previously shown not to have an inhibitory effect on HIV replication *in vitro*, similarly to what happened to heparin after desulfation [17].

The binding of heparin was also clearly dependent on the size of the oligomers, which can be interpreted both in terms of number and spatial distribution of the sulfate groups. The binding of dp12 and smaller oligomers was poorly detected, indicating that dp16 is the minimum size required for efficient binding. This size dependence seems to be an actual biological difference, rather than experimental limitation, as the readouts for the species above dp16 are clear and proportional to size, whereas below this threshold value, the readouts are in the noise level. In the protected gp120 immobilization, despite a weak binding below dp16, the instrument can still differentiate among the different GAGs. The length of HS chains in syndecans and other HSPGs is highly variable [18, 19], and therefore, a direct comparison between the minimum binding size determined by us and the lengths of the actual HSPG is difficult. However, the virions should bind more efficiently to those displaying longer and more highly sulfated chains. When gp120 interacts with CD4, the co-receptor binding site is exposed, which is also a known heparin-binding domain [8]. For that case, the minimum size for binding was determined to be dp10 [8].

The binding measured here refers directly to gp120 in its monomeric form. In the viral envelope, gp120 forms trimers. Hence, increasing the sizes of the GAG chain may increase the avidity of binding of the envelope; that is, the chain may span more than one gp120 monomer. Thus, binding to the viral envelope in an *in vivo* setting should be more stable. The avidity effect may also be a concern when measuring binding of long GAG chains (in the case of dp30 and unfractionated heparin) to the immobilized gp120 monomer, as one chain may bind more than one protein.

New strategies targeting this HIV-proteoglycan interaction are starting to appear: a syndecan-Fc hybrid molecule showed potent antiviral activity and prevented transinfection [20], and a synthetic CD4-mimetic peptide linked to an HS or an HS peptidomimetic chain also inhibited HIV-1 attachment and entry [21, 22]. These new inhibitors diversify the range of possible microbicides, increasing the probability of finding drug candidates with higher clinical potential.

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