Characterization of a Neutrophil Cell Surface Glycosaminoglycan That Mediates Binding of Platelet Factor 4*

(Received for publication, July 22, 1998, and in revised form, February 15, 1999)

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Platelet factor 4 (PF-4) is a platelet-derived α-chemokine that binds to and activates human neutrophils to undergo specific functions like exocytosis or adhesion. PF-4 binding has been shown to be independent of interleukin-8 receptors and could be inhibited by soluble chondroitin sulfate type glycosaminoglycans or by pretreatment of cells with chondroitinase ABC. Here we present evidence that surface-expressed neutrophil glycosaminoglycans are of chondroitin sulfate type and that this species binds to the tetrameric form of PF-4. The glycosaminoglycans consist of a single type of chain with an average molecular mass of ~23 kDa and are composed of ~85–90% chondroitin 4-sulfate disaccharide units type CSA (\(4\GlcA\beta1\rightarrow3\GalNAc(4-O-sulfate)\beta1\rightarrow\)) and of ~10–15% di-O-sulfated disaccharide units. A major part of these di-O-sulfated disaccharide units are CSE units (\(4\GlcA\beta1\rightarrow3\GalNAc(4,6-O-sulfate)\beta1\rightarrow\)). Binding studies revealed that the interaction of chondroitin sulfate with PF-4 required at least 20 monosaccharide units for significant binding. The di-O-sulfated disaccharide units in neutrophil glycosaminoglycans clearly promoted the affinity to PF-4, which showed a \(K_d\) ~ 0.8 μM, as the affinities of bovine cartilage chondroitin sulfate A, porcine skin dermatan sulfate, or bovine cartilage chondroitin sulfate C, all consisting exclusively of monosulfated disaccharide units, were found to be ~3–5-fold lower. Taken together, our data indicate that chondroitin sulfate chains function as physiologically relevant binding sites for PF-4 on neutrophils and that the affinity of these chains for PF-4 is controlled by their degree of sulfation.

The activation and control of polymorphonuclear granulocytes (PMN)† are known to play an essential role in host defense against microbial invaders as well as in chronic diseases.

* This work was supported in part by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 367, Projekt C4; by Swedish Medical Research Council Grant 2309; and by Polysaccharidforsknings AB (Uppsala, Sweden). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of Fellowship PE 724/1-1 from the Deutsche Forschungsgemeinschaft.

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† The abbreviations used are: PMN, polymorphonuclear cells; BSA, bovine serum albumin; CS, chondroitin sulfate; D3, dermatan sulfate; \(\Delta Di-4S, \Delta 3\) HexA1\rightarrow3GalNAc(4-O-sulfate)\beta1\rightarrow; \(\Delta Di-6S, \Delta 3\) HexA1\rightarrow3GalNAc(6-O-sulfate)\beta1\rightarrow; \(\Delta Di-diS, \Delta 3\) HexA1\rightarrow3GalNAc(6,4-O-sulfate)\beta1\rightarrow; Di-diS, disulfated disaccharide; Di-S, monosulfated disaccharide; GAG, glycosaminoglycan; GalNAc, N-acetylgalactosamine; HexA, hexuronic acid; HS, heparan sulfate; IL-8, interleukin-8; mono-diS, disulfated monosaccharide; mono-S, monosulfated monosaccharide; PBS, phosphate-buffered saline; PF-4, platelet factor 4.

‡ The nomenclature to define the various CS species is based on the notion that all variants contain a significant proportion of \(4\GlcA\beta1\rightarrow3\GalNAc(4-O-sulfate)\beta1\rightarrow\) disaccharide units. Although CSA contains no additional sulfated disaccharide unit, the other CS species do; the characteristic units are: \(4\GlcA\beta1\rightarrow3\GalNAc(4-O-sulfate)\beta1\rightarrow\) (additional sulfated substituents may occur) for CSB, \(4\GlcA\beta1\rightarrow3\GalNAc(6-O-sulfate)\beta1\rightarrow\) for CSC, \(4\GlcA(2-O-sulfate)\beta1\rightarrow3\GalNAc(6-O-sulfate)\beta1\rightarrow\) for CST, and \(4\GlcA\beta1\rightarrow3\GalNAc(4,6-O-sulfate)\beta1\rightarrow\) for CSE.

Several members of the α-chemokine family like interleukin-8 (IL-8), neutrophil-activating peptide 2, or melanoma growth stimulatory activity have been shown to act as potent activators of PMN by binding to common IL-8 receptors (1). Such binding elicits diverse biological responses such as chemotaxis, degranulation, or adhesion. PF-4, another member of the α-subgroup of the chemokine family, is released in high concentrations from activated platelets (2, 3). The functional role of PF-4 is intriguing. Highly purified PF-4 lacks any apparent biological activity for PMN but will in the presence of tumor necrosis factor α stimulate these cells to exocytose secondary granule markers or adhere tightly to different surfaces (4). These PF-4-induced functions are not elicited through binding to IL-8 receptors but by interaction with distinct binding sites different from all other chemokine receptors known so far (4, 5). The action of PF-4 on PMN was shown to be sensitive to chondroitinase ABC treatment and could be inhibited by soluble chondroitin sulfate (CS), indicating that the potential receptor is of CS proteoglycan type (5).

CSs are galactosaminoglycans composed of alternating glucuronic acid and galactosamine units (\(4\GlcA\beta1\rightarrow3\GalNAc(4-O-sulfate)\beta1\rightarrow\)), that are O-sulfated on one or both units. In contrast to the glucosaminoglycans heparin and heparan sulfate (HS), they do not contain N-sulfate groups or i-uronic acid units (except for CSB), which have been particularly implicated in protein binding to HS chains (6). The expression of glycosaminoglycans (GAGs) on neutrophils has been described previously by several authors. Pioneering work by Olsson and co-workers showed that PMN predominantly express chondroitin 4-sulfate (CSA) (7, 8), and Levitt et al. demonstrated a minor proportion of HS in these cells (9). However, as all of these analyses were done with total cell extracts, little is known about the composition and function of cell surface-expressed GAGs in PMN. Gardner and colleagues showed that the majority of metabolically \(^{35}S\)-labeled compounds occurs as proteoglycans in neutrophil granules where they may enable proper storage of granule contents or exert protective functions against cellular damage (10, 11). Here, we provide evidence that surface exposed CS chains serve as physiologically relevant receptors for PF-4 on PMN, and propose that this function is critically dependent on the content of sulfate groups.
**EXPERIMENTAL PROCEDURES**

**Materials—**BioGel P-10 (superfine) was from Bio-Rad (Sundbyberg, Sweden). Ficoll-Hypaque, Sephadex G-15, DEAE-Sephalose, Superose 6, and Superose 12 (packed 1.5 × 30 cm) for FPLC were obtained from Pharmacia (Uppsala, Sweden). Carrier-free Na$_2$SO$_4$ (1200–1400 Ci/mmol) was purchased from NEN Life Science Products (Sollentuna, Sweden). [H]$^3$Acetic anhydride (500 mCi/mmol) and n-6-[H]Hyalu-

Human PF-4 (22 Cl) was purified from releasates of supernatants of thrombin-

stimulated platelets in a three-step procedure as described previously (4). The final PF-4 preparation exceeded 99% purity and contained no protein contaminants detectable on silver-stained SDS-polyacrylamide gels, in an enzyme-linked immunosorbent assay for β-thromboglobulin antigen, or by automated N-terminal amino acid sequencing (kindly performed by Dr. A. Petersen, Department of Clinical Medicine, Forschungszentrum Borstel, Borstel, Germany).

CSA from bovine nasal cartilage, dermatan sulfate (CSB) from porcine skin, and CSC from bovine nucleus pulposus cartilage were generous gifts from Dr. Anders Malmström (University of Lund, Lund, Sweden). CSD from shark cartilage and CSE from squid cartilage were obtained from Seikagaku (Tokyo, Japan), while bovine lung heparin was received from The Upjohn Co. Human aortic HS (15) was a gift from Dr. Per Rorsman, University of Uppsala, Sweden. Heparin oligosaccharides of defined length used as size-defined standards were prepared as described previously (16). Hyaluronan fragments for the same purpose were provided by Dr. Kerstin Lidholt (University of Lund, Lund, Sweden). Ficoll-Hypaque, Sephadex G-15, DEAE-Sephacel, Superose 6, Whatman 3MM filter paper was purchased from Whatman Ltd (Maidstone, Kent, United Kingdom) and nitrocellulose filter (0.45-μm pore size) from Sartorius AG (Göttingen, Germany).

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**Indication of PF-4 and PF-4 Receptor Binding Assay—**PF-4 was iodinated using the chloramine-T method as described for IL-8 (17). To control the integrity of the labeled chemokine, iodinated PF-4 was tested for its capacity to induce an eosinophylic response in PMN (4), its ability to form tetramers (5), and its capacity to bind to different GAGs (filter binding assay, see below). No differences to unlabeled PF-4 were seen in these assays. Specific receptor binding of 1 μM [35S]PF-4 to PMN was determined as reported previously (5).

**Chemical Radiolabeling of GAGs—**Heparin was N-[$^3$H]Acetylated at free amino groups as described before (18) to a specific activity of 20,000 dpm/μg of hexuronic acid. Chondroitin sulfates (0.5 mg) were partially N-deacetylated by hydrazinolysis for 60 min at 96 °C in 1 ml of 1 M HCl. Chondroitin sulfates (1.9 mg) were then N-[$^3$H]Acetylated with 2.5 mCi of [H]$^3$Acetic anhydride, essentially as described (20). Specific activities of 8 × 10$^5$ dpm/μg for CSA, 2 × 10$^6$ dpm/μg for CSB, 6 × 10$^5$ dpm/μg for CSC, 9 × 10$^5$ dpm/μg for CSD, and 6 × 10$^5$ dpm/μg for CSE were achieved. CSA fragments resulting from a hydrazinolysis for 4 h were separated on a BioGel P-10 column (1 cm × 140 cm) equilibrated with 0.5 mM NH$_4$HCO$_3$. Pools of size-defined fragments were collected by reference to standard heparin fragments.

**Preparation and Metabolic Labeling of Human Neutrophils—**PMN cells were routinely isolated from citrated whole blood or fresh buffy coats of healthy single donors by gradient centrifugation on Ficoll-Hypaque to a purity consistently greater than 95% as described previously (21). Viability was examined by trypan blue exclusion and exceeded 98% in all experiments. Metabolic labeling was performed as described by Gardiner et al. (10). Briefly, PMN cells were washed twice with PBS and subsequently incubated at a concentration of 10$^6$ cells/ml in sulfate-free Dulbecco’s modified Eagle’s medium, supplemented with 5% fetal calf serum, 1% glutamine, and 100 μCi/mL Na$_2$SO$_4$ or 10 μCi/mL [H]$^3$Acetic anhydride for 5 h at 37 °C in humidified air. Cells were washed three times with an excess of PBS, and cell clumps were removed by trapping of cells with wet glass beads. After centrifugation, the membrane-bound GAG was dissociated from the membrane-trapped proteins in 2 M NaCl and analyzed for radioactivity in a β-scintillation counter. In some assays, binding affinities of PF-4 for different GAGs were assessed by transformation of the data according to Scatchard. As the molar amounts of PF-4 even at the lowest dosages used in the assay exceeded those of the GAGs by at least 5-fold, the amount of free PF-4 was set to total PF-4.
RESULTS

Characterization of Neutrophil Surface GAGs—In previous experiments we had shown that PMN proteoglycans responsible for PF-4 binding were rather resistant toward proteolytic digestion by trypsin or chymotrypsin (5). For this reason, a more unspecific protease (Streptomyces protease type XIV) was used for the digestion of neutrophils. In a first approach, experiments were designed to explore the time course for the elimination of PF-4 binding sites. A constant concentration of 500 μg/ml protease was used and the residual capacity for binding of iodinated PF-4 was monitored at different time-points as described (5). Binding of 1 μg/ml [125I]PF-4 to PMN at 4 °C decreased over time with 20% residual binding remaining after 10 h, and 5% residual binding after 18 h of incubation (data not shown). Therefore, the incubation time for proteolytic digestion of neutrophil PGs was extended to 36 h, when PF-4 binding was decreased to background levels. Under these conditions, about 40–50% of the total metabolically 35S-labeled material could be mobilized, while 50–60% remained cell-associated. No difference in the enzymatic activity of the primary granule marker elastase or content of the secondary granule marker lactoferrin (both determined in detergent-treated lysates) was seen between non-treated and protease-treated cells after 36 h. Furthermore, no marker protein was found in the cell-free supernatant (data not shown), indicating that neither primary nor secondary granules had leaked. These results support the notion that the integrity of granules after the proteolytic treatment was conserved such that the remaining 35S-labeled material presumably would be located in neutrophil granules (10, 11).

The released 35S-labeled surface GAGs were further purified by ion-exchange chromatography, after which the molecular size was determined by gel filtration on a Superose 6 column. PMN-GAGs eluted as a single broad peak representing molecules of a wide range of molecular sizes with an average of ~23 kDa (Fig. 1). This value corresponds to a GAG chain length of about 90 monosaccharide units (calculated with an average mass of 500 Da for a disaccharide unit), similar in size to a bovine nasal cartilage CSA preparation used as a standard. In order to characterize the GAG chains, the metabolically labeled PMN-GAGs were treated with either nitrous acid at pH 1.5 or with chondroitinase ABC and AC, respectively, followed by gel chromatography of the products. Nitrous acid treatment at pH 1.5 did not affect the elution behavior of the sample, indicating the absence of N-sulfated hexosamine residues as found in HS

![Fig. 1. Size determination of neutrophil GAGs. PMN-derived 35S-labeled GAGs (●) or N-[3H]acetyl-labeled CSA (○) (10,000 dpm each) were separated on a Superose 6 gel filtration column. Elution positions of standard, size-defined [3H]heparin fragments (1.3, 3.3, 6.6, and 8.6 kDa) and hyaluronic acid (11.9, 18.9, 30, and 43 kDa) are indicated by arrowheads. The void volume (V0) was determined with hyaluronan and the total volume (Vt) with 3H2O.](image)

![Fig. 2. Susceptibility of neutrophil GAGs to deamination by nitrous acid and to chondroitinase digestion. PMN-GAGs (15,000 dpm ~ 0.5 μg) were treated with nitrous acid at pH 1.5 (A) or digested with 0.1 unit/ml chondroitinase ABC (B) or chondroitinase AC (C) as described under “Experimental Procedures”. Digestion products (○) or untreated controls (●) were separated on a Superose 12 column.](image)
units in neutrophil surface CS appeared to be a general phenomenon as the analysis of disaccharides from two donors revealed similar disulfated disaccharide contents of 12 and 13% with minor variations between individuals (data not shown). Also, [3H]glucosamine labeling was performed to assess the amount of non-sulfated disaccharide units in the whole population. No non-sulfated disaccharides could be detected by chondroitinase ABC digestion of these preparations followed by high voltage electrophoresis, indicating that the chains are composed essentially of sulfated disaccharide units (data not shown).3

Paper chromatography of the monosulfated disaccharide fraction PI showed a single peak corresponding to the standard ΔDi-4S disaccharide (Fig. 3B), whereas no material co-migrating with the ΔDi-6S standard was seen. In order to assign the sulfation pattern of the disulfated disaccharide in fraction PI, the disaccharides were treated with mercuric acetate (26) and the resulting products were separated by high voltage electrophoresis. About 85% of the labeled material displayed an increased migration after Hg-acetate cleavage as compared with the untreated control (Fig. 3C). This peak corresponds to the disulfated monosaccharide GalNAc(4,6-OSO3), originating from the disaccharide ΔHexA1–3GalNAc(4,6-OSO3) (ΔDi-diS). The remaining 15% of the generated monosaccharides migrated slower than the original disaccharide and correspond to a monosulfated monosaccharide, originating from either ΔHexA(2-OSO3)1–3GalNAc(4,6-OSO3) (ΔDi-diS) or AΔHexA(2-OSO3)1–3GalNAc(6-OSO3) (ΔDi-diS). In summary, the neutrophil surface GAGs contain predominantly (up to 90%) chondroitin-4-sulfate disaccharide sequences, while 12–15% of the chains consists of disulfated disaccharide units, mainly due to the presence of GalNAc(4,6-disulfate residues.

PF-4 Binding to Neutrophil Surface CS Chains—In order to characterize the putative PF-4 binding sites on the CS chains, enzyme protection assays were performed. Purified 35S-labeled PMN-CS was preincubated with increasing concentrations of PF-4 and subsequently digested with chondroitinase ABC. At a concentration of 10 μM PF-4, more than 95% of the PMN CS-chains were protected against digestion with the bacterial eliminase (Fig. 4). However, a stepwise decrease of the PF-4 concentration led to a corresponding decrease in the amount of protected polysaccharide: at 1 μM PF-4 59% and at 0.2 μM only 13% of the total radioactivity remained in the high molecular weight fraction. At 0.04 μM PF-4, protection of the CS chains was completely abrogated and all of the labeled carbohydrates eluted in a second peak, representing the breakdown products. Therefore, at sufficiently high concentrations PF-4 can bind to and protect all PMN-derived CS. Corresponding control experiments performed with the bovine [3H]CSA revealed a similar PF-4-mediated protection of these chains against digestion with chondroitinase ABC. However, compared with the PMN-GAGs, the concentration of PF-4 required for complete protection was significantly higher (20 μM) indicating a lower affinity of the chemokine for the bovine CSA. Notably, decreasing the concentrations of PF-4 led to a reduction in the total amounts of protected CS chains but did not affect the size of these chains (Fig. 4). Thus, binding of PF-4 to the polysaccharide protected the entire chains from digestion but did not reveal any limited fragment that could be identified as a binding site for the chemokine.

Structural Requirements of CS-Binding to PF-4: The Importance of Chain-length and Disulfated Disaccharide Units—As PF-4 appeared to protect the entire neutrophil CS chain from lyase digestion, our next approach was to identify the minimal fragment size for PF-4 binding. Binding of size-defined, 3H-labeled CSA fragments to PF-4 was examined using a nitrocel-
chemokine was mixed with \(^{3}H\)CSA that had been partially degraded by extended hydrazinolysis (27), and the mixture was subjected to digestion with chondroitinase ABC. Analysis of the undigested \(^{3}H\)CSA preparation by gel chromatography showed a broad peak from 10.5 to 18.5 ml (Fig. 5B), representing chain lengths varying between dimers and 48-mers, with an average approximate size of a 20-mer (14.5 ml). The same material that had been treated with chondroitinase ABC in the presence of PF-4 emerged as two clearly separated peaks. The first peak, representing the protected fraction, started to elute in the same range as the untreated control, indicating full protection of the largest fragments. Fragments were protected in the same range as the untreated control, indicating full protection of the largest fragments. Fragments were protected down to \(-22\)–\(26\)-mer size. However, fractions corresponding to smaller fragment size showed a sharp decrease in radioactivity, indicating that chains shorter than \(-20\) monosaccharide units (eluting after 14.5 ml) were not protected by PF-4. These smaller fragments were found to be completely degraded by chondroitinase and eluted in the second peak, representing the breakdown products. The same labeled GAG fragments in control digestions lacking PF-4 were completely degraded and eluted exclusively in the second peak (data not shown). Taken together, the results of the two experiments indicate that PF-4 binding to CSA requires a minimal saccharide sequence of \(-20\) monosaccharide units.

The affinity of CSA to PF-4 is considered low as compared with that of heparin or HS (28). As the affinity of GAGs for chemokines appears related to the charge of the carbohydrate chain (29), we wondered whether the presence of disulfated disaccharide units in neutrophil-derived CS would influence the binding to PF-4. Filter binding assays thus were performed with labeled PMN-CS or CSA at constant concentration (\(-1\) \(\mu\)g/ml), mixed with purified PF-4 at increasing concentrations. The binding curves indicated that PMN-CS bound with higher affinity to PF-4 than did CSA (Fig. 6). Scatchard analysis of the data (Fig. 6, inset) revealed an unusual binding pattern composed of essentially two phases. Linear plots were obtained only above certain minimal concentrations of PF-4 (1.25 and 2.5 \(\mu\)M, respectively), in interactions with PMN-CS and CSA). Based on these data, PF-4 exposed to both CS types a single class of binding sites with apparent \(K_{d}\) values of \(-0.8\) \(\mu\)M for PMN-CS and \(-4.4\) \(\mu\)M for CSA, hence more than 5-fold different. However, at concentrations below 1 \(\mu\)M PF-4, the affinity of the GAG chains for the chemokine ligand decreased dramatically. Almost identical non-linear binding patterns were described for the interaction of PF-4 with binding sites on intact neutrophils, suggesting similar mode of binding of PF-4 to isolated CS and to intact cells (5). The cellular receptors were shown to preferentially bind tetrameric PF-4, which was found to occur only at concentrations exceeding 50 nM.
PF-4 Binding to Neutrophil Glycosaminoglycans

**FIG. 6.** Equilibrium binding of PF-4 to neutrophil-derived CS and to CSA. Approximately 0.2 µg of 35S-labeled PMN-CS (○) or of 3H-labeled CSA (□) were incubated with increasing concentrations of PF-4 for 2 h at 37 °C, and binding was assessed by the nitrocellulose filter assay. Inset, data were transformed according to Scatchard (dotted lines). Only values representing >5000 dpm bound for PMN-GAGs and >3600 dpm bound for CSA were used for the determination of affinity constants (solid lines). The data represent mean ± S.D. of three independent experiments, each performed in duplicate.

binding to PF-4 was considered. For this purpose, binding assays were performed with various 3H-labeled CSs from different sources and affinity constants were determined as before. Furthermore, all CS preparations were analyzed for their content of disulfated disaccharide units. Bovine intestinal heparin as well as human aortic HS served as references. As shown in Table I, all of the GAGs tested bound to PF-4, but with significantly different affinities. CSA, CSB, and CSC, which did not contain any detectable disulfated disaccharide units, showed relatively low affinities for PF-4, $K_d$ values ranging from 2.9 to 4.4 µM. However, CSD, which yielded about 8.7% ΔDi-diS upon chondroitinase digestion, bound to PF-4 with an affinity comparable to that of PMN-CS ($K_d$ values of 0.6 and 0.8 µM, respectively). Moreover, CSE, with the highest content of ΔDi-diS (30.7%), scored the lowest $K_d$: 0.3 µM. Interestingly, binding of PF-4 to HS from aorta revealed an approximately 2.5-fold lower affinity ($K_d$ ~ 2.3 µM) as compared with that of PMN-CS. By contrast, heparin, the most negatively charged carbohydrate tested, bound PF-4 with appreciably higher affinity than any of the other GAGs tested (Table I).

**DISCUSSION**

The composition of neutrophil GAGs has been investigated by several groups over the last decades. However, the GAGs expressed at the PMN surface remain poorly defined with regard to structure as well as functional role(s). The present study was initiated by our observation that PF-4 binds to a CS proteoglycan on human PMN cells (5). We therefore aimed at characterizing surface exposed GAGs and their binding to PF-4. Proteolytic release of cell surface-associated GAGs under conditions that removed all binding sites for PF-4 but conserved the macroscopic appearance of the cells resulted in the isolation of a GAG pool that contained CS, essentially of the CSA type, but no HS (Fig. 2). These findings are in accordance with earlier findings that CSA constitutes the major part of neutrophil GAGs (7, 8). Under the conditions of isolation, only about 40–50% of the total metabolically 35S-labeled material was released from the cells, whereas the rest remained cell-associated. Assessment of cellular integrity by means of marker proteins from either primary or secondary granules indicated that no leakage had occurred from these intracellular compartments. It therefore seems reasonable to assume that the remaining radioactivity would be localized intracellular, in agreement with earlier publications localizing the majority of intracellular GAGs to the granules (10, 11). Up to 25% of GAGs from total PMN extracts was identified as HS, based on susceptibility to nitrous acid treatment (9). Although we cannot exclude the presence of HS in PMN, we conclude that HS is not expressed at the cell surface and therefore does not participate in the recognition of PF-4 by these cells. Such recognition is mediated by CS chains.

**TABLE I**

| GAG type       | Content of ΔDi-diS | $K_d$ µM |
|----------------|--------------------|----------|
| PMN-CS         | 12.3 ± 1.5         | 0.8 ± 0.1|
| CSA            | None               | 4.4 ± 0.7|
| CSB            | None               | 2.9 ± 0.2|
| CSC            | None               | 4.4 ± 0.6|
| CSD            | 8.7                | 0.6 ± 0.2|
| CSE            | 30.7               | 0.3 ± 0.1|
| HS             | ND $^a$            | 2.3 ± 0.2|
| Heparin        | ND $^a$            | 0.06 ± 0.02|

$^a$ The content of disulfated disaccharides was determined by high voltage electrophoresis of labeled disaccharides prepared by complete digestion of GAGs with chondroitinase ABC as described in Fig. 3A.

$^b$ Affinity constants were determined from data of binding assays after transformation according to Scatchard. Assays were performed as described for PMN-CS and CSA in Fig. 6. The data represent mean ± S.D. of three independent experiments, each performed in duplicate.

$^c$ The data represent mean ± S.D. of results obtained from GAGs of three different healthy donors.

$^d$ ND, not determined.

Compositional analysis of the isolated CS chains indicated ~90% [→4GlcAβ1→3GalNAc4OSO$_3$]β1→α (CSA) units, in agreement with previous findings (7–9). In addition, however, we identified a significant proportion of disulfated disaccharide units, most of which contained 4,6-di-O-sulfated GalNAc units (CSE type), whereas a minor portion carried sulfate groups on both monosaccharide units.

PF-4 binds to CS as well as to heparin-related GAGs (12, 13, 28, 30, 31), and is stored as a CS proteoglycan/PF-4 complex in α-granules of platelets (32, 33). The affinity of PF-4 for different GAGs has been postulated to decrease in the order heparin >> HS >> DS > CSC > CSA (28). In previous work, PF-4 displayed the highest affinity for heparin of all the chemokines tested (13); a $K_d$ of 30 nM (12) is in fair agreement with the value (60 nM) determined in the present study. However, the postulated generalized order of affinities for GAGs needs to be modified to account for the effects of minor variations in the degree of sulfation. Although PMN-derived CS contained only ~13% di-O-sulfated disaccharides, it bound PF-4 with an affinity ($K_d$ ~ 0.8 µM) more than 5-fold higher than that of the strictly monosulfated CSA from nasal cartilage ($K_d$ ~ 4.4 µM), and more than 2-fold higher than that of HS from human aorta HS ($K_d$ ~ 2.3 µM). The positions of the additional sulfate residues appear to be less important for the increased affinity, as in CSD, with an affinity for PF-4 ($K_d$ ~ 0.6 µM) comparable to that of PMN-derived CS, the disulfated disaccharides have the GlcAβ2-OSO$_3$β1→3GalNAc(6-OSO$_3$) and not the GlcAβ1→3-GalNAc(4,6-OSO$_3$) structure as predominantly found in PMN-CS.

PF-4 is a member of the CXC chemokine family, with a three-dimensional structure very similar to that of other mem-
PF-4 Binding to Neutrophil GAGs

members of this family. The monomeric unit, consisting of a C-terminal aliphatic α-helix lying on top of a three-stranded antiparallel β-sheet (34), forms dimers and tetramers (35). Basic amino acid residues implicated with GAG binding are predominantly located in the α-helix, but also in loops of the β-sheets (12, 36), in such a way that a PF-4 tetramer will display a belt of positively charged residues around the entire molecule (37). This arrangement may help to explain some intriguing observations pertaining to the molecular dimensions of GAG-PF-4 interactions. Although a short heparin fragment molecule (37). This arrangement may help to explain some interesting observations pertaining to the molecular dimensions of GAG-PF-4 interactions. Although a short heparin fragment of ~6/8-mer size is shown to bind PF-4 (16), maximal affinity is attained only for >20-mers (16, 38). These extended sequences are believed to interact with both dimer subunits of a tetramer (38), as has been found for HS (14). The interaction between CS and PF-4 is too weak to show up at the 6/8-mer level, but is clearly evident for ~20-mer sequences (Fig. 5A), thus suggesting similar modes of binding for CS, HS and heparin. Moreover, even more extended, ~9-kDa (~40 monosaccharide units), sequences of HS were protected against heparitinase digestion in the presence of PF-4 (14). These large fragments were shown to contain sulfated domains positioned at both ends, separated by a central, mainly N-acetylated region, and would be expected to wrap around the entire circumference of a PF-4 tetramer (14). CS fragments of similar initial size were also protected by PF-4 against digestion with chondroitinase ABC (Fig. 5B). Unexpectedly, however, more extended CS chains (average ~19 kDa) were either completely degraded by the enzyme or remained seemingly intact, depending on the relative proportions of CS and PF-4 (Fig. 4). A likely explanation to this finding is that the extended CS chain interacts with more than one PF-4 tetramer, in such a manner that essentially the entire length of the chain is engaged in protein binding. Provided that sufficient amounts of PF-4 are present to saturate all CS chains in the mixture, these will be completely protected against enzymatic cleavage; unbound CS chains will be completely degraded. A HS chain will behave differently, due to its less homogeneous structure, highly sulfated domains being interspersed by essentially unsulfated sequences (39). The latter structures will be less prone to protein binding, hence protection, and will therefore be preferentially cleaved during incubation with the appropriate endoglycosidase. Composite domain structure for HS fragments interacting with cytokines have been postulated not only for PF-4 (14), but also for interferon-γ (40) and IL-8 (29).

The results of the present study are clearly relevant to the mode of action of PF-4 at the PMN cell surface. The Κₒ for PF-4 interaction with isolated PMN-CS chains (~0.8 μM) was in a range similar to that determined for the binding to intact PMN (~0.65 μM). Moreover, the binding curves obtained with whole cells (5) and with isolated CS showed similar sigmoidal shapes and non-linear Scatchard plots, indicating a decrease in the affinity for PF-4 binding sites at low concentrations of the chemokine. This phenomenon is most likely caused by the selectivity of neutrophil GAGs for binding to the tetrameric form of the chemokine. As we have shown previously, tetramerization of PF-4 takes place only at concentrations exceeding 50 nM PF-4, and in the absence of PF-4 tetramers neither binding to cellular receptors, nor functional activation of PMN is detected (5). Finally, the effects of “oversulfation” of the PMN-CS on PF-4 binding should be considered. The increase in binding strength caused by the presence of disulfated disaccharide units would seem to be of key importance in the control of PF-4 binding and PF-4-mediated cellular activation. As about half-maximal occupation of the implicated receptors on PMN is required for the induction of a measurable cellular response (4), whereas the serum concentrations of PF-4 do not exceed 1.0–2.5 μM (41), a receptor substituted simply with CSA (Kₒ ~ 4.4 μM) would hardly recruit sufficient amounts of the chemokine to mediate a cellular response. Although it seems likely that the signaling component of the PF-4 receptor is a protein constituent, receptiveness is determined by the composition of the associated CS chains. We cannot exclude that a PMN-CS proteoglycan serves as a co-receptor that is coupled to a secondary receptor function. An important step toward the elucidation of the signaling mechanism will be the isolation and characterization of the putative receptor core protein.

Acknowledgments—We thank Dr. Anders Malmström (University of Lund, Lund, Sweden) for providing CSA, CSB (dermatan sulfate), and CSC. We are also grateful to Dr. Kerstin Lindholt and Dr. Emadollin Feyzi (University of Uppsala, Uppsala, Sweden) for supplying us with size-defined hyaluronan-fragments and human aortic HS, respectively. We especially thank Prof. Ingemar Bjork (Swedish University of Agriculture Science, Uppsala, Sweden) for helping us with the interpretation of the binding data.

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