Specific Binding of the Chemokine Platelet Factor 4 to Heparan Sulfate*

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Platelet factor 4 is a tetrameric heparin binding chemokine released from the α-granules of activated platelets. In this study we show that platelet factor 4 binds with high affinity and specificity to an approximately 9-kDa sequence in heparan sulfate, which it protects from degradation by heparinase enzymes. This protected fragment is enriched in N-sulfated disaccharides and iduronate 2-O-sulfate residues, the latter being important for binding to platelet factor 4. The major structural motif of the fragment appears to consist of a pair of sulfated domains positioned at both ends separated by a central mainly N-acetylated region. On the basis of these findings, we propose a model in which the heparan sulfate fragment wraps around the ring of positive charges on platelet factor 4 with the iduronate 2-O-sulfates within the sulfated domains binding strongly to lysine clusters on opposite faces of the tetramer.

Platelet factor 4 (PF4) is a platelet-released cytokine, with a number of properties associated with inflammation and wound healing (for review see Ref. 1), some of which are thought to be due to its ability to neutralize the activities of heparin and heparan sulfate proteoglycans. PF4 has been proposed to exert procoagulant activity by preventing the formation of the stable heparin-antithrombin III-thrombin ternary complex. It also inhibits binding of heparin-binding growth factors such as basic fibroblast growth factor, vascular endothelial growth factor (splice variant 165), and transforming growth factor β1 to their receptors (3–5) and is antiangiogenic, having been shown to inhibit proliferation and migration of endothelial cells in vitro (6). Subsequent studies demonstrated the ability of PF4 in vivo to specifically bind to areas of active angiogenesis (7) and to inhibit the growth of murine melanoma and colon carcinoma, probably as a result of suppressing tumor-induced neovascularization (8, 9).

Carboxyl-terminal fragments of PF4 have been shown to retain some of the activities of whole PF4, such as blocking the interaction of bFGF with its receptor (10) and inhibiting angiogenesis (6). Two pairs of C-terminal lysines (residues 61 and 62 and residues 65 and 66) on an amphipathic α-helix in each PF4 monomer are thought to be important in binding heparin, since
guanidination of these (11) or digestion with carboxypeptidase (12) decreases binding. However, NMR studies of PF4 suggested that arginines (residues 20, 22, and 49) in other regions of the protein are also critical to the interaction with heparin, and site-directed mutation of these arginines reduced heparin binding 7-fold (13). PF4 exists mainly as a tetramer at physiologically relevant, since, following release from platelets, PF4 is thought to bind to HS proteoglycans on endothelial cells (21). In the present study we have examined the binding of PF4 to HS. The results indicate that the pattern of sulfation and spacing of the sulfated (S) domains are important for this interaction. On the basis of our findings we have proposed a model of PF4 binding to HS that may be relevant to other chemokines.

EXPERIMENTAL PROCEDURES

Materials—Platelet factor 4 was purified from human platelet concentrates (22). d-[6-3H]Glucosamine hydrochloride (20–45 Ci/mol) was obtained from NEN Life Science Products (Steventon, UK). Heparinase 1 (Flavobacterium heparinum; EC 4.4.2.7), chondroitinase ABC (Proteus vulgaris; EC 4.2.2.4), N-deacetylated N-sulfated heparin, and N-sulfated heparin were kindly provided by Dr B. Mulloy (National Institute for Biological Standards and Control, Hertfordshire, UK); 2-O- and 6-O-desulfated heparin were from Seikagaku Kogyo Co. (Tokyo, Japan). Heparinase II (F. heparinum; no EC number assigned) and heparinase III (F. heparinum; EC 4.2.2.8) were obtained from Grantam Enzymes (Aberdeen, UK). Bovine lung heparin, selectively O-sulfated heparin (approximately 68% of the 6-O-SO4 removed with most of the 2-O-SO4 present) and N-desulfated N-acetylated heparin or O-sulfated N-acetylated heparin were kindly provided by Dr B. Mulloy (National Institute for Biological Standards and Control, Hertfordshire, UK); 2-O- and 6-O-desulfated heparin were provided by Professor H. Baumann (Makromolekulare Chemie und Textilchemie, Aachen, Germany); and heparin desulfated at C-2 only was provided by Dr. B. Casu (Istituto Chemica e Biochimica, Milan, Italy). Carboxyl-reduced heparin was prepared by G. Rushton (Paterson Institute of Cancer Research, Christie Hospital, Manchester, UK) according to Taylor et al. (23). Biogel P10 and Affigel 10 were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK), Sepharose CL6B and DEAE Sephacel were purchased from Pharmacia Biotech Inc., and Heparin-agaro was purchased from Sigma (Poole, UK).

Radiolabeling and Preparation of Intact HS Chains—HS chains bio-synthetically labeled with [3H]glucosamine were prepared from nearly confluent cultures of mouse 3T3 fibroblasts, as described by Lyon et al. (24) for fetal skin fibroblast HS. To remove any remaining amino acids from the core protein, the HS chains were incubated in 50 mM NaOH, 1 M NaBH4 overnight at room temperature, and the reaction was neutralized with acetic acid. The molecular size distribution of the material
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was then analyzed by gel filtration chromatography on a Sepharose CL6B column (1 × 100 cm) eluted with 0.25 m NH4HCO3 at a flow rate of 5 ml/h.

**Filter Binding Assay**—A modified version of the filter binding assay of Maccarana et al. (25) was used. Briefly, "H-radiolabeled HS was incubated with 1 μg of PF4 plus any nonradioactive glycosaminoglycans or inhibitors for 10 min at 37 °C in 10 μl of Tris buffer (130 mM NaCl, 50 mM Tris-HCl, pH 7.3). The volume was then made up to 300 μl by the addition of Tris buffer, and the samples were drawn through buffer-equilibrated cellulose nitrate filters on a vacuum manifold. The filters were washed with 2 × 5 ml of 130 mM NaCl, 50 mM Tris-HCl, and bound material eluted with 2 × 5 ml of 250 mM NaCl, 50 mM Tris-HCl or the appropriate NaCl concentration in affinity experiments. On average greater than 99% of the radiolabeled material was removed from the filters with 2 ml of NaCl, 50 mM Tris-HCl.

To assess PF4 binding affinity for HS, Scatchard analysis of the data collected by the filter binding assay was used. The lines of best fit and graphical equations for the data were determined by Cricket Graph III Apple Macintosh computer software. The gradients of these lines are equivalent to −1/KD(1) and −1/KD(2), the x intercept for the first line represents the number of binding sites on the protein (n1), and the x intercept for the second represents n1 + n2, where n2 is the number of binding sites with K2.

**Specific Degradation of HS**—Nitrous acid degradation was performed by the low pH method of Shively and Conrad (28). Degraded samples were neutralized by the careful addition of 1 mM Na2CO3. Heparinase I and heparinase III (also known as heparitinase I) enzyme digestions were both performed with additions of 20 mU/ml enzyme in 0.1 M calcium acetate, 1 mg/ml bovine serum albumin, pH 7.0, at 30 °C for heparinase I or room temperature for heparinase III. To ensure maximum breakdown of HS, at least two additions of the enzymes were made over an 18-h period, with the final addition of heparinase III carried out at 37 °C for at least 1 h. The extent of breakdown was followed spectrophotometrically at 232 nm, or an aliquot of “H-radiolabeled digested was checked on a Biogel P10 column (1 × 100 cm) eluted with 0.25 m NH4HCO3.

**Preparation of the PF4-protected Domain (PPD) of HS**—Approximately equimolar quantities of "H-radiolabeled HS and PF4 (6 μg each) were preincubated for 10 min at room temperature before digestion by heparinase III, used at a final concentration of 0.5 mU/ml enzyme in 0.5 mM calcium acetate, 50 mM NaAc, and 0.1 mg/ml bovine serum albumin, pH 7.0, in 140 μl for about 14 h at room temperature. A further addition of 10 mU of heparinase III (in 50 μl of 0.5 mM CaCl2, 50 mM NaAc, 0.1 mg/ml bovine serum albumin, pH 7.0) was made after 8 h, and finally an additional 10 mU of enzyme were added for 2 h at 37 °C. The digest was then heated at 95 °C for 30 min to denature the enzyme and PF4. The resultant fragments were separated on a Biogel P10 column (1 × 100 cm) eluted with 0.25 m NH4HCO3, the void volume peak pooled and freeze-dried for 48 h to remove the NH4HCO3 and redissolved in distilled water. The molecular size of an aliquot was then checked on Sepharose CL6B. The sample was denatured for a further hour at 95 °C. Clear weight molecular weight saccharide fragments were precipitated by the addition of 0.3 x sodium acetate and 3 volumes of 95% (v/v) ethanol for 2 h at −70 °C and pelleted by 15-min microcentrifugation at 13,000 rpm. The pellet was rinsed in 75% ethanol, air-dried, and redissolved in distilled water. For preparation of nonradiolabeled PF4-protected HS species, fractions from the P10 and CL6B gel columns were analyzed spectrophotometrically at 232 nm.

**Affinity Chromatography**—To prepare a PF4 affinity gel column, 500 μg of human PF4 was mixed with 500 μg of heparin in 100 μl of coupling buffer (0.1 M HEPES, 80 mM NaCl, pH 7.0) and incubated for 20 min at room temperature. The PF4 was then bound to Aff-Gel 10, and the column was prepared as described for an hepatocyte growth factor affinity column by Lyon et al. (24) alongside a control column where the PF4 was omitted.

Affinity experiments were performed by application of radiolabeled HS samples in a 20 mM sodium phosphate buffer of physiological ionic strength and pH, i.e. 0.15 x NaCl and pH 7.3. The sample of HS was recirculated through the column at least five times at room temperature to maximize its opportunity to bind to PF4. The column was then washed with 25 μl of 0.15 x NaCl, 20 mM sodium phosphate, pH 7.3, followed by 2.5 ml of each of NaCl concentrations from 0.2 to 1.5 x in 0.05 x increments. 0.5-ml fractions were collected and monitored for radioactivity.

To determine PF4 binding affinity for heparin, 5 μg of PF4 was loaded onto a column containing 500 μl of heparin-agarose gel slurry that had been equilibrated in 50 mM Tris, 0.13 M NaCl buffer. The PF4 was eluted with 4-ml steps of 50 mM Tris buffers containing NaCl concentrations ranging from 0.1 to 1.8 x in 0.1 x increments. 1-ml fractions were collected and monitored by spectroscopy at 260-nm absorbance.

**RESULTS**

**Interaction of PF4 with HS**—To examine the affinity of PF4 for HS, a filter binding assay was used (25). 1 μg of human PF4 was incubated with various quantities of "H-labeled murine 3T3 fibroblast HS; the solution was passed through a cellulose nitrate filter, which adsorbs PF4 or the PF4-HS complex but not HS itself; and the proportion of bound material was determined by filter binding as described under “Experimental Procedures.” Two lines (solid lines) have been fitted to the curve using the software package Cricket Graph III, where Lb, Lf, and M represent the concentrations (in nM) of bound HS, free HS, and PF4 tetramers, respectively. The dashed lines indicate the intersects of the curves with the x and y axes, which are equivalent to the number of binding sites (n) for HS on the PF4 tetramer and m/KD. The inset shows a semilog plot of the saturation curve of the same data. B, competitive inhibition of "H-labeled HS chains binding to PF4 by unlabeled glycosaminoglycans. The inhibition curves are given for bovine lung heparin (□□□□□□□□), porcine intestinal mucosal HS (●●●●●●), and dermatan sulfate (××××××). Error bars (S.E. = S.D./number of samples) are visible where they exceed the symbol size. Each experiment was repeated at least three times.

![Fig. 1. Filter binding assay of the interaction of PF4 with HS and other glycosaminoglycans](http://www.jbc.org/)}
as a negative cooperativity curve, where increases in the number of molecules of HS binding in a range from one to six, due to increased HS:PF4 ratios, correlates with a decrease in the binding affinity.

PF4 elution from a heparin affinity column was also determined to allow comparison with published studies of PF4 and other heparin-binding proteins. As expected from previous reports (11), PF4 applied at physiological ionic strength bound strongly to heparin and eluted between 1.1 and 1.6 m, with a median of 1.35 m, in a stepwise NaCl concentration gradient (data not shown).

Glycosaminoglycan Specificity of PF4 Binding—A range of glycosaminoglycans were examined for their ability to competitively inhibit binding of 1 μg (99 nm) of 3H-radiolabeled murine 3T3 fibroblast HS to 1 μg (107 nm) of PF4 tetramers, by the filter binding assay to give an insight into binding requirements (Fig. 1B). Although the heavily sulfated heparins were found to be the strongest inhibitors of binding, HS was a significantly more efficient inhibitor than the other glycosaminoglycans tested. Bovine lung heparin and porcine intestinal mucosal heparan sulfate inhibited binding at lower concentrations than the structurally distinct dermatan sulfate, with IC50 values of 0.03, 0.2, and 2 μg, respectively (Fig. 1B). Similarly, porcine intestinal mucosal heparin had an IC50 of 0.04 compared with 1.2 μg for chondroitin sulfate (data not shown).

Binding of an equivalent amount of bFGF to HS, which is known to be a strong interaction (28), was inhibited by 0.2 μg of bovine lung heparin in the same assay.

Requirement of Particular Chemical Groups—Competition studies using a range of specifically modified heparins were carried out to determine which groups were important in the interaction with PF4. Replacement of the N-sulfates on the glucosamines with N-acetyl groups had no effect on the inhibitory activity of heparin, the IC50 remained at 0.04 ± 0.01 μg (mean ± S.E. to 2 decimal places). Partial 6-O-desulfation of heparin (66% depletion of 6-sulfate groups) only slightly increased the IC50 to 0.06 ± 0.01 μg, whereas removal of the 2-O-sulfates from the IdoAs increased the IC50 3-fold to 0.12 ± 0.00 μg, suggesting that the latter groups may be of particular importance in the interaction with PF4. The carboxyl groups also seem to be involved in the interaction as carboxyl reduction of the uronic acids increased the IC50 2-fold to 0.08 ± 0.00 μg. The IC50 for 2- and 6-O-desulfated heparin was particularly high at 5 μg, 125-fold higher than the intact polysaccharide, whereas the IC50 of totally desulfated heparin was so high it was not obtainable, being above 140 μg.

Effects of Specific Enzyme Scission—To identify domains important for PF4 binding, competition studies were carried out with porcine mucosal HS digested by the enzymes heparinase I and/or heparinase III that cleave the polysaccharide in different structural regions. Heparinase I acts in the N-sulfated regions and specifically cleaves disaccharides that contain 2-O-sulfated iduronate i.e. GlcNSO3(±6-OSO3)1,4IdoA2-OSO3 (29). By contrast, heparinase III cleaves GlcA-containing disaccharides (29), principally GlcNAc/GlcNSO3α1,4GlcA, that are present in regions of low sulfation and does not contiguously N-sulfated sequences that are enriched in IdoA. The binding to PF4 of HS fragments produced by heparinase I or heparinase III was significantly decreased by comparison with native HS. The IC50 values increased by approximately 3-fold in each case, to 0.52 ± 0.10 μg for heparinase I-digested HS and to 0.62 ± 0.06 μg for heparinase III-digested HS compared with 0.22 ± 0.00 μg for the intact HS. This indicates that both N-sulfated (S-domains) and N-acetylated regions of the HS chain are important for binding. Digestion of HS with both heparinase I and heparinase III together had a significant additive effect, further increasing the IC50 to 1.30 ± 0.05 μg.

Isolation of the PF4 Binding Site on HS (Protection Assay)—Since the PF4 binding site appeared to overlap both N-acetylated and N-sulfated regions of HS, the binding domain could not be isolated from fragments produced by scission with either heparinase I or heparinase III. Therefore, a protection assay was used in which PF4 was included in a heparinase digest to prevent cleavage of the binding site. Initial results (data not shown) demonstrated that the sizes of HS binding fragments protected by PF4 during digestion with either combined heparinases I and III or heparinase III alone were not significantly different, so most experiments were carried out with heparinase III. Native polyacrylamide gel electrophoresis of porcine HS/PF4 mixtures confirmed that no aggregates containing more than 1 PF4 tetramer were formed at the 1:1 ratios used (results not shown). A prolonged digest was carried out to ensure that all the heparan sulfate was digested apart from fragments with strong binding affinity for PF4. When the fragments (i.e. S-domains) from heparinase III breakdown of HS in the absence of PF4 were compared by gel filtration on a high
resolution Biogel P10 column with profiles for PF4-protected HS (Fig. 2, A and B) the most striking difference was the presence of an obvious peak in the void volume of the PF4-protected digest (Fig. 2B). This was surmised to be the PPD. Gel filtration chromatography of PPD on Sepharose CL-6B revealed a radioactive fraction, the peak maximum of which eluted with a mean $K_{av}$ (from several experiments) of 0.65 (Fig. 2C), equivalent to a mass of 9.3 $\pm$ 1.2 kDa by reference to the published calibration of Wasteson (30). This is equivalent to about 21 disaccharides, assuming an average of 450 Da/disaccharide. By comparison, the intact fibroblast HS had a $K_{av}$ of 0.35, equivalent to approximately 40 kDa (Fig. 2C). A similar size fragment (10.7 kDa) was isolated from porcine intestinal mucosal HS protected by PF4.

It was important to establish that PPD was indeed a PF4 binding fragment. Elution of intact HS and PPD from PF4 by a range of NaCl concentrations was examined by the filter binding assay, and the profiles were found to be similar, with both binding at up to 0.6 M NaCl (Fig. 3A). For comparison to PPD, fragments of decasaccharide size and above from a heparinase III digest of HS in the absence of PF4 were isolated, and the salt concentration at which they eluted from PF4 was tested in the filter binding assay. Half the amount of these single S-domains bound to PF4 compared with PPD, and they were completely eluted by 0.3 M NaCl (not shown), suggesting that they have significantly less affinity for PF4 than PPD.

For further comparison between PPD and intact fibroblast HS, their relative affinities for a PF4 Affi-Gel column were compared. The majority of the bound intact HS and PPD eluted between 0.2 M and 0.4 M NaCl, again with similar elution profiles (Fig. 3, B and C). No further elution was seen from the remainder of the 0.05 M stepwise gradient taken up to 1.5 M NaCl. The apparent affinity of polysaccharide for the PF4 Affi-Gel may be less than with the filter binding due to the physical restrictions of PF4 being bound to the gel. Metabolically labeled HS prepared from bovine aortic endothelial cells exhibited slightly lower affinity for PF4 eluting mainly between 0.2 and 0.3 M NaCl (data not shown). A control column did not exhibit any binding of HS (Fig. 3B, dotted line) or PPD above 0.2 M NaCl.

Analysis of specific degradations of HS and PPD by gel filtration chromatography. $^3$H-Labeled HS (----) and PPD (-----) were degraded by exhaustive treatment with low pH nitrous acid (A), heparinase III (B), and heparinase I (C) as described under “Experimental Procedures.” The digests were analyzed by chromatography on a Biogel P10 column. Distinct oligosaccharide peaks are labeled according to the number of monosaccharide units.
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The $^3$H profiles in Fig. 4 were used to calculate the percentages of glycosidic bonds susceptible to cleavage by nitrous acid, heparinase I, or heparinase III in PPD and intact fibroblast HS, the latter of which is shown in parentheses.

| Specific cleavage reagent | Linkage specificity | Distribution of cleaved linkages | Total linkages cleaved |
|--------------------------|---------------------|---------------------------------|------------------------|
| HNO$_3$                  | GlcNSO$_3$HexA      | 35 (17)                         | 61 (39)                |
| Heparinase I             | GlcNSO$_3$(IdoA2-OSO$_3$) | 14 (5)                        | 21 (13)                |
| Heparinase III           | GlcNR-GlcA          | 36 (56)                         | 53 (68)                |

* These disaccharide structures can also contain O-sulfate at appropriate C-2 and C-6 positions. Only O-sulfates absolutely essential to the reagent specificity are detailed. Abbreviations used are: HexA, α-1-idurionate or β-D-glucuronate; R, H or SO$_3$.

** Contiguous linkages give rise to disaccharide products. Alternate susceptible linkages possess single intervening resistant linkages and therefore give rise to tetrasaccharide products. Spaced susceptible linkages have two or more intervening resistant linkages, thereby giving hexasaccharide and larger oligosaccharide products.

This indicates a significant increase in contiguous N-sulfated disaccharides by comparison with the original HS. The overall level of N-sulfation was 61% in PPD compared with 39% in HS (Table I).

Heparinase III scission provides data on the size range of the S-domains in HS, elucidating in part the arrangement of the contiguous N-sulfated disaccharides identified by nitrous acid hydrolysis. Heparinase III yielded distinctive patterns of scission of PPD and HS (Fig. 4B). There was a notable depletion of heparinase III-susceptible linkages in PPD (53% compared with 68% in HS), with significant enrichment of hexa- and octasaccharide S-domains (Fig. 4B, Table I).

There was also a notable increase in heparinase I-susceptible IdoA 2-O-sulfate containing disaccharides in PPD compared with the original HS (Fig. 4C, Table I). Heparinase I cleavage released 3-fold more disaccharides and twice the amount of tetrasaccharides from PPD than from the original HS. Since there were no midsized fragments released (Fig. 4C), the IdoA 2-O-sulfates must be toward the ends of PPD. As described earlier (Fig. 2C) on CL6B Sepharose, heparinase I-treated PPD eluted in a peak that overlapped with the position of PPD itself but with a peak maximum at 1 kDa smaller, confirming that only the end termini of PPD must have been cleaved. Since the use of partially desulfated heparins had indicated that IdoA 2-sulfate residues are important in the binding of polysaccharide to PF4, the affinity of heparinase I-treated PPD for PF4 was investigated. This had no binding apparent above 0.3 M NaCl in the filter binding assay (data not shown) and hence much lower affinity for PF4 than PPD, which bound up to 0.6 M (Fig. 3A).

The prevalence of PPD of particular sized fragments in heparinase III and I digestions and nitrous acid hydrolyses can be rationalized in the form of a major structural motif, with some permissible variations, that represents this enzyme-protected binding region for PF4 in HS. Such a structure is depicted in Fig. 5A in the form of a model where the important IdoA 2-sulfate disaccharides are within short S-domains (three or four disaccharides) at opposite ends of the approximately 21-disaccharide PPD. The S-domains are separated by an extended region of relatively low sulfation that contains the cleavage sites for heparinase III (Fig. 5A). A model of PF4-PPD complex is also shown (Fig. 5B).

** DISCUSSION **

We have demonstrated by Scatchard analysis that a 1:1 ratio of HS to PF4 tetramer binds with a $K_d$ of 16 nM, compared with 30 nM previously reported for the more highly sulfated heparin (12). Our results from competitive inhibition studies demonstrated the importance of S-domains in binding of HS to PF4, since heparinase I digestion caused a 3-fold increase in the IC$_{50}$.

However S-domains alone, isolated by heparinase III digestion, were unable to reproduce the binding properties of the parent molecule. These findings are in contrast to those for bFGF and for the extracellular matrix molecule, fibronectin, where the optimum HS binding sites appear to be contained within one extended sulfated domain (28, 31). The use of partially desulfated heparins emphasized the importance of 2-O-sulfate groups present on the IdoAs for binding to PF4, with possibly some requirement for 6-O-sulfation of the glucosamines, but N-sulfate groups were not necessary. Since IdoA 2-sulfate residues are also essential for bFGF binding to HS (28), PF4 may inhibit bFGF activity by competing for an overlapping site. However it cannot be ruled out that removal of these groups could have an effect on the secondary structure of HS that could be detrimental to its binding to PF4.

The foregoing results on the effects of enzyme scission and competing polysaccharide on the binding of HS to PF4 are not consistent with an interaction that is dependent solely on charge density. Reduction of net charge on PF4 mutants also affected heparin binding less than would be expected if charge alone were responsible for the interaction (13), and it was suggested that aggregation of the monomers contributed to the strength of interaction (32). This suggestion has been taken into account in our model of the PF4-PPD complex (Fig. 5B).

The most striking difference with most other previously characterized binding sites on HS is the unusually large molecular mass (9.3 kDa, equivalent to 21 disaccharides) of the PF4-binding fragment (PPD) isolated from murine fibroblast HS. This correlates with earlier reports showing a requirement of at least 10-kDa heparin fragments for optimum binding to this protein (15, 16). The disaccharide composition and the depolymerization profiles after specific enzyme and chemical scission (Fig. 4, Table I) were used to determine key structural features of PPD shown in the model in Fig. 5A.

The heparinase III depolymerization profile (Fig. 4B) indicates that murine 3T3 fibroblast HS in common with human fibroblast HS (33) largely consists of blocks of 3–7 N-sulfated disaccharides (i.e. the S-domains) spaced apart by extended N-acetylated sequences. The 21-disaccharide PPD contains on average 13 N-sulfated disaccharides, 61% of which are contiguous, i.e. present in S-domains (Table I). Therefore, four of the predominant hexa- and octasaccharide S-domains (Fig. 4B) may be accommodated in PPD (Fig. 5A). Heparinase I cleavage of the 9.3-kDa PPD only reduced its size by approximately 1 kDa (Fig. 2C) and released di- and tetrasaccharides (Fig. 4C, Table I), indicating that these susceptible IdoA 2-sulfate-containing S-domains (34) may be toward the ends of PPD (Fig. 5A). The four S-domains have been depicted as pairs at each end of the PPD model (Fig. 5A), although in a minority of cases each or either pair are merged into a single larger S-domain (10–14 saccharides), which were present in some PPD fragments (Fig. 4B). The proportion of GlcA-containing disaccharides in PPD (Fig. 4B, Table I) represents seven contiguous
The large size of PPD favors the hypothesis that it wraps around PF4 in wounds should promote the chemotactic action of PF4 on the subendothelial fibroblasts and weakly responsive neutrophils (1). The specificity of the interaction of PF4 with HS and the specificity of the interaction strongly suggest important biological and biochemical properties of the HS-PF4 complex. At sites of vascular injury where platelet activation occurs, the concentration of PF4 can rise to 170 μM (1) (compared with 0.45 nM in unstimulated plasma), strongly favoring its binding with vascular HS. This should effectively block the binding of anticoagulant proteases such as antithrombin III to sequences within the S-domains of HS and thus favor blood coagulation. Furthermore, the accumulation of PF4 in wounds should promote the chemotactic action of PF4 on the subendothelial fibroblasts and weakly responsive neutrophils (1). The specificity of the interaction of PF4 with HS may enable it to bind preferentially to the HS at sites of active angiogenesis (7), which might account for its ability to inhibit angiogenesis and suppress tumor growth (8, 9). The model of an extended region of HS tethered by peripheral S-domains encircling subunits of PF4 could be a common mechanism of interaction with multimeric cytokines (35) and strengthens the view that the spacing of the S-domains within HS may be as critical as argued (36) in creating the model of PF4 structure was greatly appreciated.

A protection study similar to that described here was used to identify the binding site in heparan sulfate for the interferon-γ dimer (35). A protected fragment of 10 kDa was isolated that shares some structural features with PPD. However, the interferon-γ-binding domain (named IDP) apparently contained only one S-domain at each end of the fragment and a greater prevalence of central linkages cleaved by heparinase III. In common with the PPD structure described in the present study, the peripheral S-domains in IDP were essential for the binding activity of interferon-γ.

The large size of PPD favors the hypothesis that it wraps around the PF4 tetramer, neutralizing the ring of positive charges (Fig. 5B), as has been postulated for heparin (13, 17, 36, 37). 34 saccharides running perpendicular to the α-helices around PF4 have been proposed from x-ray crystallographic models to be the minimum number to form salt links with all four lysines of each monomer (17). The 42 saccharides of PPD may be accommodated into a perpendicular model with the extra central N-acetylated disaccharides looping out slightly from the tetramer. The paired nature of the S-domains, which contain the important IdoA 2-sulfate residues, at the ends of PPD, favors this model, since they would be in a position to interact with the lysine clusters in the pairs of antiparallel α-helices at opposite sides of the PF4 tetramer (Fig. 5B). Other important cationic residues, such as arginines 20, 22, and 46 (13), which encircle PF4, would interact with the GlcA and occasional N-sulfated groups in the central region of PPD (Fig. 5B).

A number of the basic residues in the ring of charges on PF4 are conserved within the family of intercellular chemokines (17), which includes interleukin-8, β-thromboglobulin, neutrophil-activating protein-1, interferon-γ-induced protein-10, and gro-α, -β, and -γ, emphasizing their likely physiological importance for chemokine function. Similarity in the x-ray crystallographic structure of interleukin-8 to PF4 infers that HS may also encompass interleukin-8, although it may recognize a different structural domain from PF4 (38). The distinctive nature of PF4 binding to HS and the specificity of the interaction strongly suggest important biological and biochemical properties of the HS-PF4 complex. At sites of vascular injury where platelet activation occurs, the concentration of PF4 can rise to 170 μM (1) (compared with 0.45 nM in unstimulated plasma), strongly favoring its binding with vascular HS. This should effectively block the binding of anticoagulant proteases such as antithrombin III to sequences within the S-domains of HS and thus favor blood coagulation. Furthermore, the accumulation of PF4 in wounds should promote the chemotactic action of PF4 on the subendothelial fibroblasts and weakly responsive neutrophils (1). The specificity of the interaction of PF4 with HS may enable it to bind preferentially to the HS at sites of active angiogenesis (7), which might account for its ability to inhibit angiogenesis and suppress tumor growth (8, 9).

The presence of both sulfated and nonsulfated domains in PPD and the high level of 2-O-sulfation supports the sequence requirements deduced from the competitive inhibition studies.

A protection study similar to that described here was used to identify the binding site in heparan sulfate for the interferon-γ dimer (35). A protected fragment of 10 kDa was isolated that shares some structural features with PPD. However, the interferon-γ-binding domain (named IDP) apparently contained only one S-domain at each end of the fragment and a greater prevalence of central linkages cleaved by heparinase III. In common with the PPD structure described in the present study, the peripheral S-domains in IDP were essential for the binding activity of interferon-γ.

The large size of PPD favors the hypothesis that it wraps around the PF4 tetramer, neutralizing the ring of positive charges (Fig. 5B), as has been postulated for heparin (13, 17, 36, 37). 34 saccharides running perpendicular to the α-helices around PF4 have been proposed from x-ray crystallographic models to be the minimum number to form salt links with all four lysines of each monomer (17). The 42 saccharides of PPD may be accommodated into a perpendicular model with the extra central N-acetylated disaccharides looping out slightly from the tetramer. The paired nature of the S-domains, which contain the important IdoA 2-sulfate residues, at the ends of PPD, favors this model, since they would be in a position to interact with the lysine clusters in the pairs of antiparallel α-helices at opposite sides of the PF4 tetramer (Fig. 5B). Other important cationic residues, such as arginines 20, 22, and 46 (13), which encircle PF4, would interact with the GlcA and occasional N-sulfated groups in the central region of PPD (Fig. 5B).

A number of the basic residues in the ring of charges on PF4 are conserved within the family of intercellular chemokines (17), which includes interleukin-8, β-thromboglobulin, neutrophil-activating protein-1, interferon-γ-induced protein-10, and gro-α, -β, and -γ, emphasizing their likely physiological importance for chemokine function. Similarity in the x-ray crystallographic structure of interleukin-8 to PF4 infers that HS may also encompass interleukin-8, although it may recognize a different structural domain from PF4 (38). The distinctive nature of PF4 binding to HS and the specificity of the interaction strongly suggest important biological and biochemical properties of the HS-PF4 complex. At sites of vascular injury where platelet activation occurs, the concentration of PF4 can rise to 170 μM (1) (compared with 0.45 nM in unstimulated plasma), strongly favoring its binding with vascular HS. This should effectively block the binding of anticoagulant proteases such as antithrombin III to sequences within the S-domains of HS and thus favor blood coagulation. Furthermore, the accumulation of PF4 in wounds should promote the chemotactic action of PF4 on the subendothelial fibroblasts and weakly responsive neutrophils (1). The specificity of the interaction of PF4 with HS may enable it to bind preferentially to the HS at sites of active angiogenesis (7), which might account for its ability to inhibit angiogenesis and suppress tumor growth (8, 9). The model of an extended region of HS tethered by peripheral S-domains encircling subunits of PF4 could be a common mechanism of interaction with multimeric cytokines (35) and strengthens the view that the spacing of the S-domains within HS may be as critical for protein binding as the sulfation patterns (39).

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