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# Differential binding of chemokines to glycosaminoglycan subpopulations

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**Background:** Specificity in leukocyte trafficking is likely to depend on sequential interactions between various cell-type-specific leukocyte adhesion molecules, such as selectins and integrin ligands, and leukocyte-activating factors. A major class of leukocyte-activating factors, the chemokines, are soluble polypeptides that bind glycosaminoglycans, the polysaccharide components of cell-surface and extracellular-matrix proteoglycans. It has been suggested that cell-surface glycosaminoglycans of the heparin/heparan sulfate class mediate the presentation of chemokines to leukocytes by vascular endothelial cells. We investigated the possibility that specificity exists in the recognition of particular heparin/heparan sulfate structures by chemokines, by studying the binding of four members of the chemokine superfamily to heparin and heparan sulfate.

**Results:** Using affinity co-electrophoresis we found that interleukin-8 preferentially bound a subfraction of

heparin that also showed increased affinity for melanoma growth stimulating activity (also known as MGSA, GRO or GRO $\alpha$ ). This same subfraction of heparin, however, was not significantly preferentially bound by platelet factor 4 or neutrophil activating factor-2. Subsequent analysis of the three-dimensional structures of these chemokines indicated that their ability to discriminate among heparin subspecies correlates with the presence of paired glutamic acid residues within the putative glycosaminoglycan-binding site of the chemokine. This observation led to predictions about the relative affinities of heparan sulfate for interleukin-8 and platelet factor 4, predictions that were confirmed by further binding assays.

**Conclusion:** Chemokines can bind selectively to subsets of heparin/heparan sulfate glycosaminoglycans, raising the possibility that glycosaminoglycans participate in determining the specificity of leukocyte recruitment *in vivo*.

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## Background

Leukocyte recruitment is a highly regulated process that is thought to involve at least three sequential steps: selectin-mediated leukocyte rolling on the vascular endothelium with only transient adhesion, followed by leukocyte activation, which in turn results in cell-surface changes that enable the cell to engage in tight, integrin-mediated adhesion. The selective recruitment of specific types of leukocytes into different tissue sites has been suggested to arise through the use of varying combinations of selectins, leukocyte-activating factors and integrins deployed in specific situations *in vivo* [1]. Although the multi-step model described above is supported by both *in vitro* [2] and *in vivo* [3] studies, the high degree of specificity observed in leukocyte recruitment *in vivo* cannot be fully accounted for by the diversity of the known adhesion and activation molecules, suggesting that additional factors are involved.

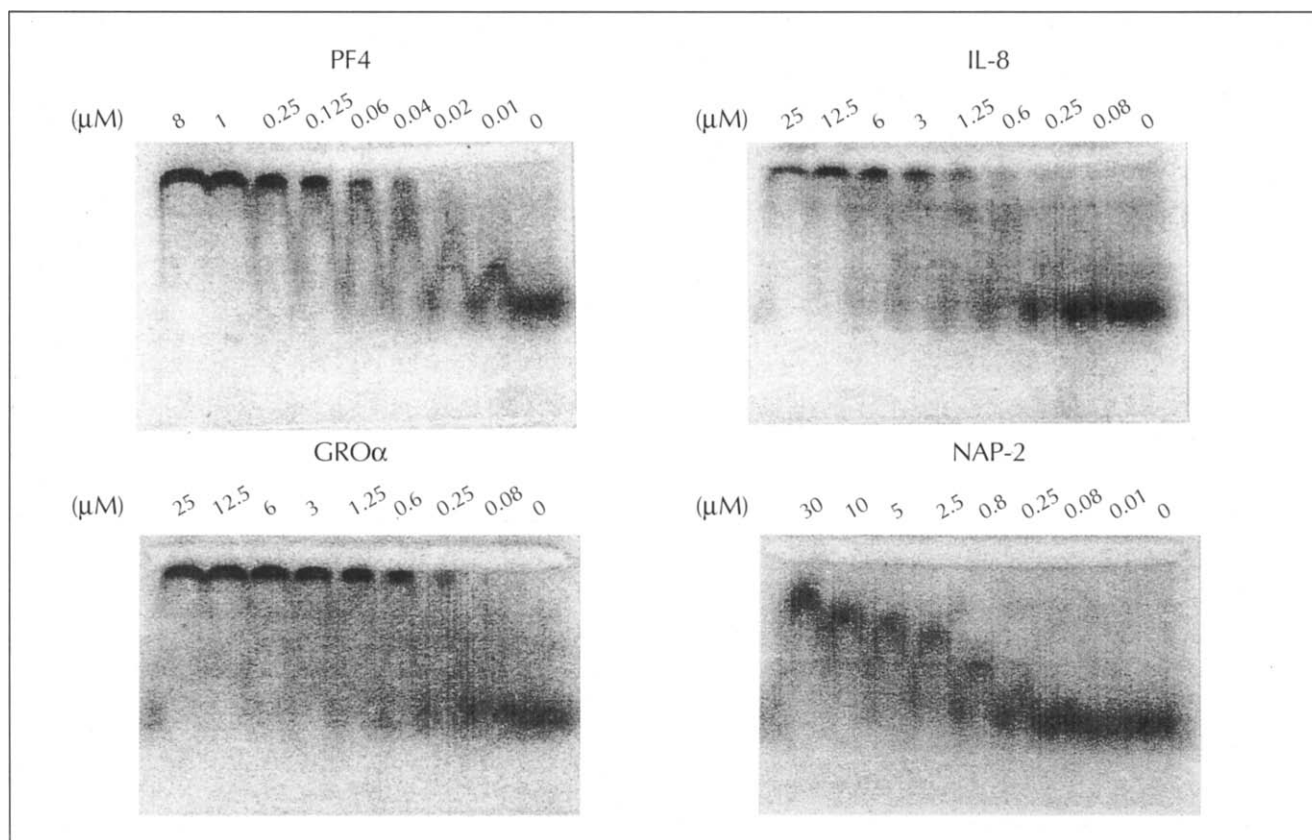
Recently, much attention has focused on the possible role in leukocyte activation of a group of proteins called the chemokines, or intercrines, which show potent leukocyte-activating and/or chemoattracting properties both *in vitro* and *in vivo* [4,5]. Although the chemokines are a large family of structurally related proteins (about

15 are known), there is considerable overlap in both the types of chemokine to which any given type of leukocyte responds, and the types of leukocyte upon which any given chemokine acts.

Intriguingly, it has been observed that the types of leukocyte recruited by administering chemokines *in vivo* are, in some cases, more restricted than had been predicted by *in vitro* assays [6]. This phenomenon suggests that, *in vivo*, other molecules increase the specificity of chemokine action. One likely mechanism is the selective association of chemokines with tissue sites that are already specialized for the recruitment of particular types of leukocyte. For example, the chemokine interleukin-8 (IL-8), which primarily recruits neutrophils, appears to bind preferentially to the endothelium of postcapillary venules, the site at which neutrophil recruitment predominantly occurs [7]. In contrast, macrophage inflammatory peptide-1 $\beta$  (MIP-1 $\beta$ ), which primarily recruits lymphocytes, is found preferentially associated with the high endothelial venules of lymph nodes [8].

One class of molecules likely to play a role in mediating tissue-specific associations of chemokines is the glycosaminoglycans. Chemokines are known to bind to

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**Fig. 1.** Analysis by affinity co-electrophoresis of binding of heparin to chemokines.  $^{125}\text{I}$ -labelled heparin was subjected to electrophoresis in four gels, each of which contained a single chemokine — PF4, NAP-2, IL-8 or GRO $\alpha$  — incorporated into nine distinct lanes. Chemokine concentrations ( $\mu\text{M}$ ) were varied systematically from lane to lane, and are indicated above each gel. The series of peaks, increasing from right to left, represent the progressive retardation of heparin mobility in the presence of increasing amounts of each chemokine. The valleys between each peak are due to the presence of chemokine-free agarose between each lane, and serve to help align the peaks with the lanes. From such electrophoretograms, the apparent  $K_d$  can be estimated from the protein concentration at which heparin is half-maximally retarded [15]. Note that, for IL-8 and GRO $\alpha$ , there is a substantial range of protein concentrations at which the migrating heparin is broadly smeared, suggesting non-homogeneity of binding [16].

glycosaminoglycans, especially heparin [9], and it has been suggested that heparin-related (heparan sulfate) glycosaminoglycans on endothelial cell surfaces localize and present chemokines to selectin-bound leukocytes [1]. This concept has been supported by recent studies suggesting that chemokines may be bound to cell-surface heparan sulfate *in vitro* [10] and *in vivo* [8,11]. Glycosaminoglycans may also modify chemokine activity [12].

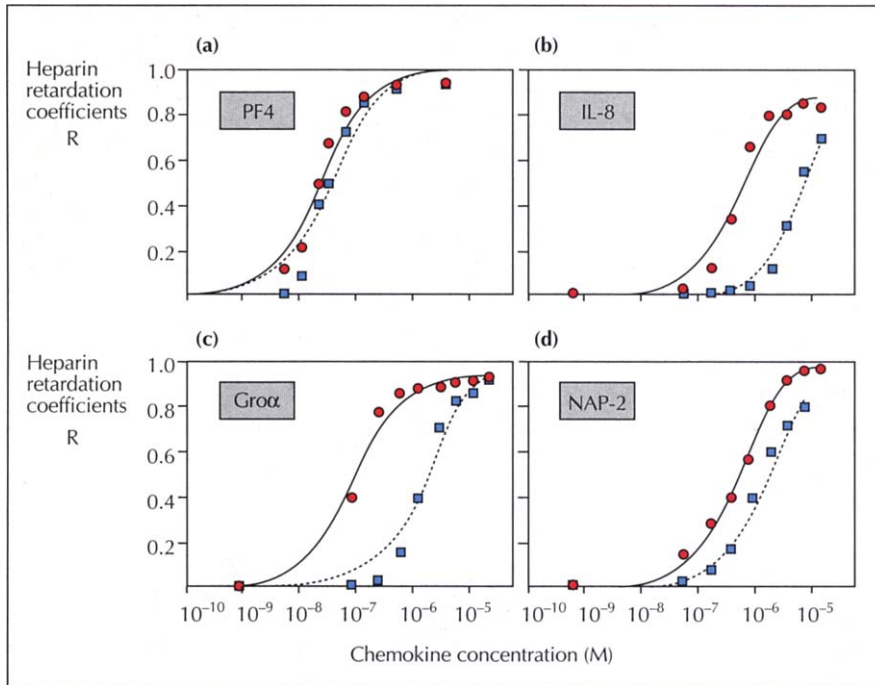
Given that glycosaminoglycan structures — even within the heparan sulfate family — can vary dramatically among tissues [13], it has been further hypothesized that subtle differences among glycosaminoglycans are responsible for the localization of particular chemokines to specific tissue locations *in vivo* [14]. However, for this hypothesis to be tenable, chemokines would need to be able to distinguish among subtly different glycosaminoglycan structures and/or sequences, and to do so in a way that differs from one chemokine to the next. We demonstrate that both of these assumptions are valid. In addition, we use information about the known three-dimensional structures of chemokines to make predictions about the structural basis of selective glycosaminoglycan binding.

## Results and discussion

Affinity co-electrophoresis [15,16] was used to study the binding of glycosaminoglycans of the heparin and heparan sulfate family to the following four members of the C-X-C chemokine ( $\alpha$ -chemokine) subfamily: interleukin-8 (IL-8), melanoma growth stimulating activity or GRO (GRO $\alpha$ ), platelet factor four (PF4) and neutrophil activating peptide-2 (NAP-2) [4]. All of these molecules bind to immobilized heparin under physiological conditions (see Materials and methods), and all but PF4 potently chemoattract and activate neutrophils [17]. PF4 and a precursor form of NAP-2, platelet basic protein, are stored in the  $\alpha$ -granules of platelets and released upon activation. IL-8 and GRO $\alpha$  are synthesized by various types of cell in response to stimulation with proinflammatory cytokines such as IL-1 and tumor necrosis factor- $\alpha$ .

### Binding of heparin to chemokines

Figure 1 shows the results of electrophoresing through agarose a radiolabeled low molecular weight fraction of porcine intestinal heparin ( $\leq 6000$ ) in the presence of different concentrations of each of the four chemokines (see Materials and methods). Apparent dissociation



**Fig. 2.** Binding of affinity-fractionated heparin to chemokines. Preparative affinity co-electrophoresis with 1  $\mu$ M IL-8 was used to separate a slower migrating (putative low-affinity heparin) from a faster migrating (putative high-affinity heparin) population of heparin chains. These two fractions were then tested for binding to PF-4, IL-8, GRO $\alpha$  and NAP-2, as described in Fig. 1. From the autoradiograms obtained, heparin retardation coefficients (R) within each lane were determined [15] and are plotted against chemokine concentration, in moles per liter, in each lane. Red circles, high-affinity heparin; blue squares, low-affinity heparin. Smooth curves represent non-linear, least-squares fits to the equation:  $R = R_{\infty} / (1 + K_d/[protein])$  [15,16]. For each curve, the apparent  $K_d$  is the chemokine concentration at which R is half-maximal.

constants ( $K_d$ ) were calculated from patterns of heparin mobility as described in [15], and reflect the protein concentration at which heparin mobility was half-maximally shifted. The molecular weights used in these calculations were based on a homotetrameric molecule for PF4 and homodimers for the other three chemokines. The values obtained were  $K_d = 5.5 \times 10^{-6}$  M for IL-8,  $9.1 \times 10^{-7}$  M for NAP-2;  $2.5 \times 10^{-7}$  M for GRO $\alpha$ ; and  $2.7 \times 10^{-8}$  M for PF4. Thus, of the four, PF4 bound most tightly to heparin and IL-8 the least.

Careful inspection of the gels in Figure 1 shows that, for IL-8 and GRO $\alpha$ , the migrating heparin front becomes diffuse at chemokine concentrations near the respective  $K_d$ , whereas, for PF4 and NAP-2, migrating heparin remains in a coherent band at all the protein concentrations. Failure of the heparin front to form a tight band suggests binding heterogeneity; that is, that the heparin fraction includes multiple species with affinities substantially higher and lower than the average  $K_d$ .

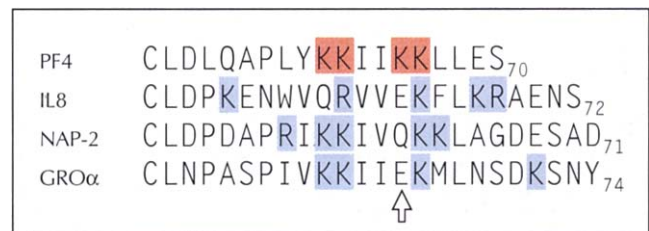
**Affinity-fractionation of heparin on IL-8, and the binding of heparin fractions to other chemokines**

To verify that IL-8 was able to bind preferentially to a subpopulation of heparin, radiolabeled heparin was subjected to electrophoresis through a single-lane preparative affinity co-electrophoresis gel containing  $10^{-6}$  M IL-8. Fractions were then obtained from the gel to represent the 15% of the heparin that was most strongly retarded in mobility (putative high-affinity heparin) and the 30% of the heparin that was most mobile (putative low-affinity heparin).

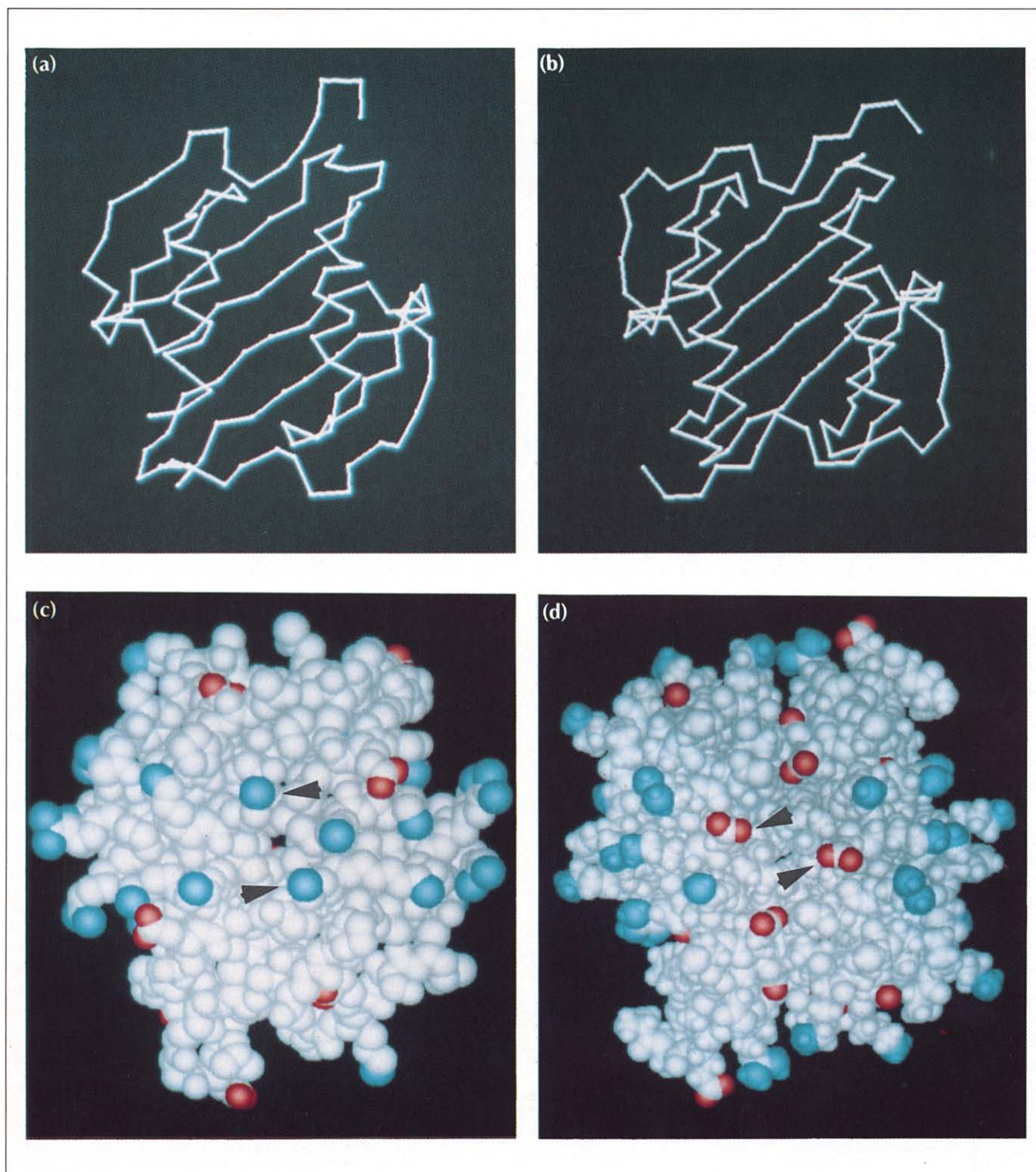
The high-affinity and low-affinity heparin fractions were re-tested for binding to all four chemokines (Fig. 2). As expected, IL-8 bound the high-affinity fraction of

heparin more tightly — about 16-fold more tightly — than the low-affinity fraction ( $K_d = 8.2 \times 10^{-7}$  M compared to  $1.3 \times 10^{-5}$  M, for high-affinity and low-affinity respectively). GRO $\alpha$  also showed marked preferential binding to the high-affinity fraction ( $K_d = 8.4 \times 10^{-8}$  M compared to  $2.0 \times 10^{-6}$  M, a difference of 24-fold). In contrast, PF4 showed only slightly stronger binding (1.6-fold) to the high-affinity fraction ( $K_d = 2.1 \times 10^{-8}$  M compared to  $3.4 \times 10^{-8}$  M). NAP-2 also bound the high-affinity fraction only slightly better (2.3-fold) than the low-affinity fraction ( $K_d = 8.6 \times 10^{-7}$  M compared to  $2.0 \times 10^{-6}$  M).

In addition to showing that all four chemokines bind heparin with affinities in the range exhibited by other proteins that interact with glycosaminoglycans *in vivo* [15,18], these results also demonstrate that two



**Fig. 3.** Comparison of the carboxyl termini of the four chemokines examined in this study [4]. Amino acids are presented using the single letter code; the sequences are aligned according to the last cysteine residue in each. A tyrosine had been incorporated at the carboxyl terminus of the GRO $\alpha$  used in these studies to permit radiolabeling (see Methods and materials) and did not affect glycosaminoglycan binding (data not shown). Basic residues are highlighted in blue and the four lysines of PF4 that are implicated in heparin binding (see text) are shown highlighted in red. The glutamic acid residue of IL-8 and GRO $\alpha$  that correlates with their ability to show preferential heparin binding (see text) is arrowed.



**Fig. 4.** Comparison of the three-dimensional structures of PF4 and IL-8. These structural representations were created using the program 'Promodeler I' (New England Biographics) with structural coordinates obtained from the Brookhaven Protein Data Bank. Only two of the four identical subunits of PF4, the A and B subunits [24], are shown here, as the AB dimer of PF4 is the structural equivalent of the IL-8 dimer. The crystal structure of the PF4 AB dimer determined to a resolution of better than 3.0 Å (X Zhang, L Chen, DP Bancroft, CK Lai, TE Maione unpublished results) is shown in (a) and (c). The solution structure of IL-8 determined by two-dimensional NMR [25] is shown in (b) and (d). (a) and (b) show the  $\alpha$ -carbon backbone traces for PF4 and IL-8 respectively, the two  $\alpha$ -helices in each representation are above the plane of the six-stranded  $\beta$ -sheet. In each case, one of the  $\alpha$ -helices and three of the  $\beta$ -strands of the  $\beta$ -sheet are contributed by each of the two subunits, and the carboxyl terminal  $\alpha$ -helices align with each other in anti-parallel fashion across the homodimer interface. In the space-filling models for PF4 (c) and IL-8 (d), all atoms, with the exception of hydrogens for PF4, are shown, with the charged atoms for all basic (Arg, Lys, His) and acidic (Glu, Asp) residues in color. Oxygens bearing a net negative charge (side-chain carboxylate oxygens for Glu and Asp) are shown in red and nitrogens capable of bearing a net positive charge ( $\epsilon$ -amino nitrogens for Lys, guanidino nitrogens for Arg, and ring nitrogens for His) are shown in blue. All other atoms are white. The Glu residues at position 63 of IL-8, which correlate with binding selectivity as discussed in the text, are indicated by the arrowheads in (d).

chemokines, IL-8 and GRO $\alpha$ , exhibit substantial selectivity in their binding to heparin species (16-fold and 24-fold, respectively). It is noteworthy that even the affinity-fractionated high-affinity and low-affinity heparin species exhibit some 'smearing' when subjected to electrophoresis through gel lanes containing appropriate concentrations of IL-8 or GRO $\alpha$  (data not shown), suggesting that, within these fractions, subpopulations exhibiting even greater differences in affinity may exist. Interestingly, the fact that fractions of heparin, isolated by their differences in affinity for IL-8, also differed strongly in affinity for GRO $\alpha$  implies that IL-8 and GRO $\alpha$  both bind preferentially to a similar heparin subpopulation.

### Relationships between chemokine structure and glycosaminoglycan selectivity

Figure 3 shows the sequence of the carboxyl terminus of PF4 aligned with homologous sequences in IL-8, GRO $\alpha$  and NAP-2. Mutagenesis studies have established that four lysine residues (Lys 61, Lys 62, Lys 65, and Lys 66) in this region are required for heparin binding [19]. In the three-dimensional structure of PF4, these residues are clustered in a group of eight on the solvent-exposed face of the homodimer; additional basic groups, contributed by other regions of the PF4 molecule, also lie on either side of this central lysine cluster (Fig. 4a, c). Altogether, these residues form a polybasic band that stretches around three faces of the molecule, and that has been proposed to constitute the heparin-binding domain of PF4 [20].

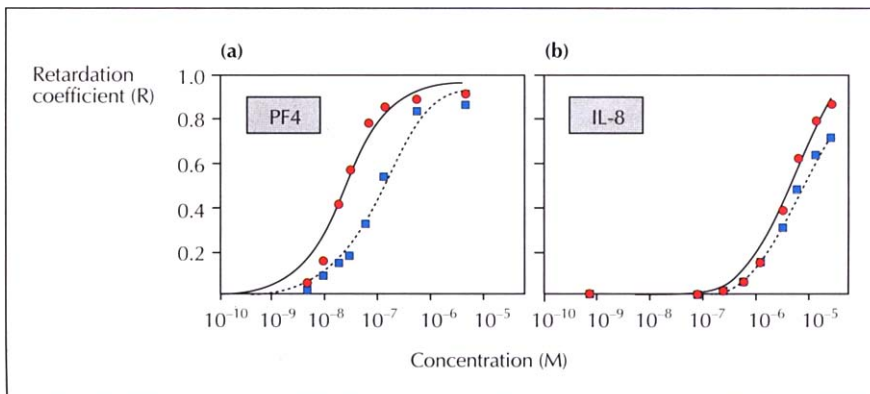
In IL-8, as in PF4, the carboxyl terminus has also been implicated in heparin binding [12]. Moreover, the carboxyl terminus of IL-8, GRO $\alpha$ , and NAP-2 can be aligned with PF4, and each contains several basic residues (Fig. 3). In the three-dimensional structure of the IL-8 homodimer, a pair of basic residues of each monomer (equivalent to Lys 62 and Lys 66 of PF4) forms a solvent-exposed cluster of four basic residues that, together with residues on adjoining faces of the molecule (Fig. 4b, d), creates a polybasic band similar to that seen in PF4. There is no other collection of positively charged residues in IL-8 that suggests itself as a likely alternative heparin-binding domain. Strikingly, in IL-8 but not in PF4, this band is interrupted by four negatively-charged residues (Fig. 4). The two negative

charges that lie directly in the center of the band are contributed by glutamic acid (Glu 63), at a position occupied by lysine (Lys 65) in PF4.

Interestingly, as shown in Figure 3, the corresponding residue (Glu 64) in GRO $\alpha$  is also acidic, whereas it is neutral in NAP-2 (Gln 61). Although an acidic residue within a heparin-binding site might be expected to decrease the affinity for heparin, it is noteworthy that the affinity of GRO $\alpha$  for heparin is 3.6-fold higher than that of NAP-2 (Fig. 1). On the other hand, both of the chemokines that have an acidic residue at this position exhibit substantial binding selectivity for a subpopulation of heparin, whereas those that lack the acidic residue exhibit little such selectivity. Moreover, no other amino-acid position is occupied by the same, or similarly charged, amino acids in GRO $\alpha$  and IL-8, and by oppositely charged or uncharged amino acids in NAP-2 and PF4.

These observations suggest that the presence of a pair of acidic residues within the heparin-binding site of a chemokine may play a role in its discrimination between different heparin species. A plausible explanation is that the acidic residues interfere, by like-charge repulsion, with the binding of some, but not other, heparin molecules. In particular, one might predict that heparin species with a short region of undersulfation would be more readily accommodated by IL-8 and GRO $\alpha$  than heparin species that are uniformly and highly sulfated. In contrast, PF4 and NAP-2 would not be expected to bind preferentially to heparin species with interrupted sulfation.

The prediction that IL-8 and GRO $\alpha$  select for heparin molecules with gaps in sulfation is intriguing in view of the structure of heparan sulfate, which is synthesized by the same polymerization and modification reactions as heparin but is modified much less extensively. As this results in an overall level of sulfation that is usually much lower than that of heparin, glycosaminoglycan-binding proteins usually bind heparan sulfate molecules substantially less strongly than they bind heparin (A.D.L., unpublished observations). Recently, however, Gallagher and co-workers [13,21] have shown that heparan sulfate molecules have a domain structure, in which blocks of highly sulfated (heparin-like)



**Fig. 5.** Binding of heparan sulfate to PF4 and IL-8. Samples of heparan sulfate and low molecular weight heparin were subjected, in parallel, to affinity co-electrophoresis in the presence of PF4 and IL-8. Gels were prepared and visualized as in Fig. 1, and retardation coefficients calculated and plotted as in Fig. 2; red circles, heparin; blue squares, heparan sulfate. The data indicate that PF4 binds heparan sulfate substantially less strongly than it binds heparin. In contrast, IL-8 binds both heparan sulfate and heparin with about the same affinity.

disaccharides alternate with regions of very low sulfation. One might predict that the structure of heparan sulfate would favor binding to IL-8 and GRO $\alpha$ , such that the observed affinity of heparan sulfate for these chemokines would be higher than expected.

This prediction was tested directly, by carrying out affinity co-electrophoresis using radiolabeled heparan sulfate from bovine intestinal mucosa (Fig. 5). When PF4 was examined, the binding of heparan sulfate was found to be weaker than the binding of heparin ( $K_d = 1.12 \times 10^{-7}$  M compared to  $2.7 \times 10^{-8}$  M, a difference of 4.1-fold), a result that fits with the idea that heparan sulfate, being undersulfated, contains weaker protein-binding sites or fewer binding sites per molecule. For IL-8, in contrast, heparan sulfate and heparin bound equally well ( $K_d = 5.5 \times 10^{-6}$  M). These results imply that at least some features of the heparan sulfate structure selectively improve its binding to IL-8.

To test definitively whether it is the gaps in sulfation of heparan sulfate that are responsible for this effect will require experiments using structurally defined heparan sulfate fragments [22]. In addition, as the present study examines the binding of chemokines to soluble glycosaminoglycans, it remains to be assessed whether or not the immobilization of heparan sulfate chains on cell surfaces has additional, as yet unknown, effects on the binding of chemokines. In any case, the possibility that IL-8 is specifically attuned to structural features of heparan sulfate raises the intriguing possibility that there may exist *in vivo* some heparan sulfate species that bind IL-8 with even higher affinity than any of the glycosaminoglycans tested here. It is known that, in at least one case, the tissue localization of a particular protein-binding sequence on heparan sulfate — the antithrombin-binding sequence — correlates with the localization of its protein ligand [23]. Consistent with this view, it has already been speculated that the preferential association of IL-8 with post-capillary venules in skin and connective tissue is the consequence of the specific localization of appropriate proteoglycans [7,11].

## Conclusions

The results presented here demonstrate that certain chemokines are able to interact preferentially with subsets of glycosaminoglycans of the heparin/heparan sulfate family. Moreover, for each chemokine the presence or absence of heparin species-selectivity correlates with the presence or absence, respectively, of an amino-acid determinant in the primary structure of the putative glycosaminoglycan-binding sites of chemokine. These findings are consistent with the view that glycosaminoglycans play an important role in the physiology of chemokines. Moreover, they suggest that tissue-specific variations in the structure of glycosaminoglycans could provide a molecular basis for the selective association of certain chemokines with distinct regions of the vascular endothelium [8,11]. In this way, glycosaminoglycans could play a key role in

adding specificity to the activation step of leukocyte recruitment [1].

## Materials and methods

### Reagents

Chemokines were obtained from Repligen Corporation. IL-8 was the 72-amino-acid (monocyte) form of the protein whereas NAP-2 and PF4 were the 71-amino-acid and 70-amino-acid naturally occurring forms, respectively. GRO $\alpha$  was the 73 amino-acid natural sequence except that a tyrosine residue was incorporated at the carboxyl terminus (74 amino acids) to permit radiolabeling in further studies. The incorporation of this extra residue did not alter the ability of the protein to bind heparin, nor did it affect receptor binding. All four chemokines bound to a heparin-agarose column in 0.25 M NaCl. IL-8 and NAP-2 were eluted from the column at 0.6–0.7 M NaCl, GRO $\alpha$  eluted at about 0.75–0.8 M, and PF4 required 1.2–1.5 M NaCl for elution from the column.

Heparin from porcine intestinal mucosa (Sigma) was derivatized with tyramine and radioiodinated as previously described in [16]. This material was then subjected to gel filtration, first on a Sephadex G-25 column to remove free isotope and then on Sephadex G-100 column to size fractionate the labeled heparin. A sample of molecular weight < 6 000 D was used the studies described here.

Bovine intestinal mucosa heparan sulfate (Sigma) was derivatized with fluorescein isothiocyanate, which couples to the short peptides that are attached to the reducing end of proteolytically derived glycosaminoglycan chain, and iodinated as described in [15]. Free isotope was removed by chromatography on a Sephadex G-25 column. No attempt was made to size fractionate the heparan sulfate preparation.

### Affinity co-electrophoresis

Samples of low molecular weight  $^{125}$ I-heparin were subjected to electrophoresis through 1% agarose gels containing lanes in which chemokines had been previously incorporated at various concentrations (see Fig. 1 legend). Molecular weights were calculated on the basis of PF4 being a tetramer and the other three chemokines being dimers. The electrophoresis buffer was 0.1 M sodium acetate, 50 mM MOPSO, 0.5% CHAPS, pH 7.0. Chemokines were resuspended at twice the desired final concentrations, mixed with an equal volume of molten, 37°C, low-gelling-temperature agarose in electrophoresis buffer, and immediately introduced into preformed lanes [15]. Following introduction of radioiodinated heparin (about  $10^4$  c.p.m. per sample), and electrophoresis, gels were dried with forced warm air, and the labeled material visualized using a Phosphorimager (Molecular Dynamics).

### Affinity fractionation of heparin

A gel containing a single lane in which IL-8 was present at a concentration of 1  $\mu$ M was used to preparatively fractionate low molecular weight  $^{125}$ I-heparin into two populations [16]. The putative high affinity fraction, containing about 15% of input heparin, consisted of material that was strongly retarded by the IL-8; the putative low affinity fraction consisted of material that was weakly retarded (containing about 30% of input heparin). The two fractions from the sliced agarose gel were melted at 65°C and quickly brought to a final concentration of 6 M urea to delay gelation. These

fractions were then retested for binding to chemokines using analytical affinity co-electrophoresis with 3 000–5 000 c.p.m. loaded per gel.

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